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(54) Title: GENE CODING FOR HELIOMICINE AND USE THEREOF

(54) Titre: GENE CODANT POUR L'HELIOMICINE ET SON UTILISATION

(57) Abstract

The invention concerns heliomicine, a DNA sequence coding for heliomicine, a vector containing it for transforming a host organism and the transformation method. The invention concerns heliomicine as medicine in particular for treating fungal infections. More particularly it concerns the transformation of plant cells and plants, the heliomicine produced by the transformed plants ensuring their resistance to diseases, in particular diseases of fungal origin.

(57) Abrégé

La présente invention a pour objet l'héliomicine, une séquence d'ADN codant pour l'héliomicine, un vecteur la contenant pour la transformation d'un organisme hôte et le procédé de transformation. L'invention concerne l'utilisation de l'héliomicine à titre de médicament, en particulier pour le traitement des infections fongiques. L'invention concerne plus particulièrement la transformation des cellules végétales et des plantes. L'héliomicine produite par les plantes transformées leur conférant une résistance aux maladies, en particulier d'origine fongique.

GENE ENCODING HELIOMICINE AND ITS USE

The subject of the present invention is a new cysteine-rich peptide called heliomicine, its use as a medicament and the compositions containing it, a DNA 5 sequence encoding this peptide, a vector containing it for the transformation of a host organism and the method of transforming the said organism.

The invention relates more particularly to the transformation of plant cells and of plants, the 10 heliomicine produced by the transformed plants conferring on them resistance to diseases, in particular of fungal origin.

There is currently an increasing need to make plants resistant to diseases, in particular fungal 15 diseases, in order to reduce or even avoid having to use treatments with antifungal protection products, in order to protect the environment. One means of increasing this resistance to diseases consists in transforming plants so that they produce substances 20 capable of providing their defence against these diseases.

In the field of human health, opportunistic fungal infections exist for which no truly effective treatment currently exists. In particular, this is the 25 case for certain serious invasive mycoses which affect hospital patients whose immune system is suppressed following a transplant, a chemotherapy or HIV

infection. Compared with the antimicrobial agent arsenal, the current range of antifungal agents is very limited. A real need therefore exists to characterize and develop new classes of antifungal substances.

5 Various substances of natural origin, in particular peptides, are known which exhibit bactericidal or fungicidal properties, in particular against the fungi responsible for plant diseases. However, a first problem consists in finding such

10 substances which not only can be produced by transformed plants, but which can still preserve their bactericidal or fungicidal properties and confer them on the said plants. For the purposes of the present invention, bactericidal or fungicidal is understood to mean both the actual bactericidal or fungicidal

15 properties and the bacteriostatic or fungistatic properties.

Cysteine-rich peptides are also known which exhibit bactericidal or bacteriostatic activities, but 20 which do not exhibit fungicidal or fungistatic activity. Another problem consists in finding a cysteine-rich peptide which exhibits a high fungicidal or fungistatic activity compared with the peptides of the state of the art.

25 Heliomicine is a peptide isolated from the haemolymph of the lepidopteron *Heliothis virescens* which exhibits fungicidal activity against the fungi

responsible for plant diseases and the fungi of human or animal pathology. After having first synthesized the gene for heliomicine, it was also found that it could be inserted into a host organism, such as a yeast or a 5 plant, so as to express heliomicine and either produce purified or nonpurified heliomicine, or confer on the said host organism properties of resistance to fungal diseases, providing a particularly advantageous solution to the problems set out above.

10 The subject of the invention is therefore first heliomicine, its use as a medicament or in agrochemistry for the protection of plants, the compositions comprising it, a nucleic acid fragment encoding heliomicine, a chimeric gene comprising the 15 said fragment encoding heliomicine as well as heterologous regulatory elements at the 5' and 3' positions which can function in a host organism, in particular in yeasts or plants and a vector for transforming the host organisms containing this 20 chimeric gene, and the transformed host organism. It also relates to a transformed plant cell containing at least one nucleic acid fragment encoding heliomicine and a plant resistant to diseases containing the said cell, in particular which is regenerated from this 25 cell. It finally relates to a method of transforming plants to make them resistant to diseases into which a gene encoding heliomicine is inserted by means of an

appropriate vector. It finally relates to a method of preparing heliomicine by transformed host organisms.

Heliomicine is understood to mean according to the invention any peptide comprising essentially the 5 peptide sequence of formula (I) below,

Xaa-Cys-Xab-Cys-Xac-Cys-Xad-Cys-Xae-Cys-Xaf-Cys-Xag

(I)

10 in which:

Xaa is -NH₂ or a peptide residue comprising from 1 to 10 amino acids, preferably from 1 to 6 amino acids,

15 Xab is a peptide residue comprising from 1 to 10 amino acids, preferably 10,

Xac is a peptide residue of 3 amino acids,

Xad is a peptide residue comprising from 1 to 9 amino acids, preferably 9,

20 Xae is a peptide residue comprising from 1 to 7 amino acids, preferably 7,

Xaf is a peptide residue of 1 amino acid, and

Xag is -OH or a peptide residue comprising from 1 to 5 amino acids, preferably 1 or 2 amino acids.

25 According to a preferred embodiment of the invention, Xaa comprises at least one basic amino acid, and/or Xad comprises at least one basic amino acid.

Advantageously, Xad comprises 1, 2, 3 or 4 basic amino

acids.

Advantageously, Xad represents the following peptide sequence -Lys-Xad'-Xad"-Gly-His-, in which Xad' represents a peptide residue of 1 basic amino acid and 5 Xad" represents a peptide residue comprising from 0 to 5 amino acids, preferably 5.

Basic amino acids are understood to mean more particularly according to the invention the amino acids chosen from lysine, arginine or homoarginine.

10 Preferably, Xad represents the following peptide sequence -Lys-Arg-Arg-Gly-Tyr-Lys-Gly-Gly-His- or Leu-Leu-Arg-Gly-Tyr-Lys-Gly-Gly-His-.

According to another preferred embodiment of the invention, Xac comprises at least one acidic amino 15 acid, preferably one.

Advantageously, Xac represents the following peptide sequence -Asn-Xac'-Xac"-, in which Xac' represents a peptide residue of 1 amino acid, and Xac" represents a peptide residue of 1 acidic amino acid.

20 Acidic amino acid is understood to mean according to the invention any amino acid comprising on a side chain an organic acid function, more particularly a carboxylic acid preferably chosen from glutamic acid (Glu) or aspartic acid (Asp).

25 Preferably, Xac represents the following peptide sequence -Asn-Gly-Glu- or Ala-Ala-Glu-.

Advantageously,

Xaa represents the following peptide sequence Xaa'-Gly-Xaa"- in which Xaa' represents NH₂ or a peptide residue comprising 1 to 9 amino acids, preferably 1 to 5 amino acids, and Xaa" represents a peptide residue comprising

5 at least one amino acid, preferably chosen from Leu, Ile, Val, Pro, Ser or Thr, and/or

Xab represents the following peptide sequence -Val-Xab'-Asp-, in which Xab' represents a peptide residue comprising from 0 to 8 amino acids, preferably 8,

10 and/or

Xae represents the following peptide sequence -Gly-Xae'-Asn-, in which Xae' represents a peptide residue comprising from 0 to 5 amino acids, preferably 5, and/or

15 Xaf represents one of the following amino acids -Trp-, Phe, Leu, Ile or Val and/or

Xag represents the following peptide sequence -Glu-Xag', in which Xag' represents OH or a variable residue having a sequence comprising from 1 to 4 amino acids,

20 preferably 1 amino acid.

According to a more preferred embodiment of the invention, Xaa represents the following peptide sequence NH₂-Asp-Lys-Leu-Ile-Gly-Ser- or NH₂-Ala-Ala-Ala-Ala-Gly-Ser-, and/or Xab represents the following peptide sequence -Val-Trp-Gly-Ala-Val-Asn-Tyr-Thr-Ser-Asp-, and/or Xae represents the following peptide sequence -Gly-Ser-Phe-Ala-Asn-Val-Asn-, and/or Xaf

represents the following amino acid -Trp- and/or Xaa
represents the following peptide sequence -Glu-Thr-OH
or -Arg-Thr-OH.

According to a more preferred embodiment of
5 the invention, the heliomicine is the peptide
represented with its coding sequence by the sequence
identifier No. 2 (SEQ ID NO 2). The same sequence is
described, corresponding to amino acids 6 to 49 of the
sequence identifier No. 1 (SEQ ID NO 1) with a
10 different coding sequence.

The NH₂-terminal residue may exhibit a post-
translational modification, for example an acetylation,
likewise the C-terminal residue may exhibit a post-
translational modification, for example an amidation.

15 Peptide sequence comprising essentially the
peptide sequence of general formula (I) is understood
to mean not only the sequences defined above, but also
such sequences comprising at either of their ends, or
at both ends, peptide residues necessary for their
20 expression and targeting in a host organism. Host
organism is understood to mean any organism comprising
at least one cell, whether microorganisms, in
particular a yeast or a bacterium, or alternatively
plant cells or alternatively higher organisms such as
25 plants.

This may be in particular a "peptide-
heliomicine" fusion peptide whose cleavage by the

enzymatic systems of the host organism allows the release of heliomicine, heliomicine being defined above. The peptide fused with heliomicine may be a signal peptide or a transit peptide which makes it

5 possible to control and orient the production of heliomicine in a specific manner in a portion of the host organism, such as for example the cytoplasm, the cell membrane, or in the case of plants in a particular type of cell compartment or of tissues or in the

10 extracellular matrix.

According to one embodiment, the transit peptide may be a signal for chloroplast or mitochondrial homing, which is then cleaved in the chloroplasts or the mitochondria.

15 According to another embodiment of the invention, the signal peptide may be an N-terminal signal or "prepeptide", optionally in combination with a signal responsible for retaining the protein in the endoplasmic reticulum, or a peptide for vacuolar homing

20 or "propeptide". The endoplasmic reticulum is the site where the operations for processing the protein produced, such as for example the cleavage of the signal peptide, are performed by the "cellular machinery".

25 The transit peptides may be either single, or double, and in this case optionally separated by an intermediate sequence, that is to say comprising, in

the direction of transcription, a sequence encoding a transit peptide of a plant gene encoding a plastid localization enzyme, a portion of sequence of the N-terminal mature part of a plant gene encoding a 5 plastid localization enzyme, and then a sequence encoding a second transit peptide of a plant gene encoding a plastid localization enzyme, as described in application EP 0 508 909.

As transit peptide useful according to the 10 invention, there may be mentioned in particular the signal peptide of the tobacco PR-1 α gene described by Cornelissen et al., represented with its coding sequence by the sequence identifier No. 2, in particular when heliomicine is produced by plant cells 15 or plants, or the precursor of factor Mat a1 when heliomicine is produced in yeasts.

The fusion peptide "MF α 1/heliomicine" with the five residues of the propeptide of factor MF α 1 (Ser-Leu-Asp-Lys-Arg), which are situated at the 20 N-terminal position, and its coding sequence are part of the present invention, described in particular by the sequence identifier No. 1 (SEQ ID NO 1), corresponding to amino acids 1 to 49.

The "PR-1 α signal peptide-heliomicine" fusion 25 peptide and its coding sequence are also part of the present invention, described in particular by the sequence identifier No. 3 (SEQ ID NO 3).

The fusion peptide comprising the signal peptide of the maize polygalacturonase PG1 gene fused with heliomicine "PG1 signal peptide/heliomicine" is represented with its coding sequence by the sequence 5 identifiers Nos. 18 and 20 (SEQ ID NO 18 and SEQ ID NO 20).

According to a preferred embodiment of the invention, the cysteine residues of the peptide of formula (I) form at least one intramolecular disulphide 10 bridge, preferably three disulphide bridges. According to a preferred embodiment of the invention, the disulphide bridges are established between the cysteine residues 1 and 4, 2 and 5, and 3 and 6.

Heliomicine is a peptide which is 15 particularly active against fungi and yeasts, and may as such be used preventatively or curatively to protect various organisms against fungal attacks. The present invention therefore relates to heliomicine as a medicament. It also relates to the use of heliomicine 20 for the treatment of plants against fungal attacks, by applying heliomicine directly to the said plants.

The present invention also relates to a composition comprising heliomicine and an appropriate vehicle. The first quality of the appropriate vehicle 25 is not to substantially degrade the heliomicine in the composition, and not to reduce the bactericidal and fungicidal properties of the heliomicine. This

composition may be a cosmetic composition and in this case the appropriate vehicle is cosmetically acceptable (suitable in addition for application to the skin or the exoskeleton), or a pharmaceutical composition for a 5 therapeutic use and in this case the appropriate vehicle is pharmaceutically acceptable, appropriate for administration of heliomicine by the topical route per os or by injection, or alternatively an agrochemical composition and in this case the appropriate vehicle is 10 agrochemically acceptable, appropriate for application to plants or in the vicinity of plants, without damaging them.

The present invention also relates to a nucleic acid fragment, in particular DNA, natural or 15 synthetic, encoding the heliomicine defined above, including the "peptide-heliomicine" fusion peptide defined above. It may be according to the invention a fragment which is synthesized or which is isolated from the lepidopteron *Heliothis*, or alternatively a derived 20 fragment, suitable for the expression of heliomicine in the host organism where the peptide will be expressed. The nucleic acid fragment may be obtained according to standard isolation and purification methods, or alternatively by synthesis according to the customary 25 methods of successive hybridizations of synthetic oligonucleotides. These techniques are in particular described by Ausubel *et al.*

According to the present invention, "nucleic acid fragment" is understood to mean a nucleotide sequence which may be of the DNA or RNA type, preferably of the DNA type, in particular double-stranded.

According to one embodiment of the invention, the nucleic acid fragment encoding heliomicine comprises the DNA sequence described by bases 16 to 147 of the sequence identifier No. 1 (SEQ ID NO 1), or by 10 the sequence identifier No. 2 (SEQ ID NO 2), in particular the coding portion of this sequence corresponding to bases 1 to 132, a homologous sequence or a sequence complementary to the said sequence.

According to another embodiment of the 15 invention, the nucleic acid fragment encoding the "peptide-heliomicine" fusion peptide comprises the DNA sequence described by the sequence identifier No. 1 (SEQ ID NO 1) or that described by the sequence identifier No. 3 (SEQ ID NO 3), in particular the 20 coding portion corresponding to bases 3 to 224, or that described by the sequence identifier No. 18 (SEQ ID NO 18), in particular the coding portion corresponding to bases 7 to 205, a homologous sequence or a sequence complementary to the said sequences.

25 "Homologue" is understood to mean according to the invention a nucleic acid fragment exhibiting one or more sequence modifications relative to the nucleotide

sequence described by the sequence identifiers Nos. 1, 2 or 3 and encoding heliomicine or the "peptide-heliomicine" fusion peptide. These modifications may be obtained according to the customary mutation

5 techniques, or alternatively by choosing the synthetic oligonucleotides used in the preparation of the said sequence by hybridization. In the light of the multiple combinations of nucleic acids which may lead to the expression of the same amino acid, the differences

10 between the reference sequence described by the sequence identifiers Nos. 1, 2 or 3 and the corresponding homologue may be substantial, all the more so since small-sized DNA fragments are involved which can be produced by chemical synthesis.

15 Advantageously, the degree of homology will be at least 70% compared with the reference sequence, preferably at least 80%, more preferably at least 90%. These modifications are generally neutral, that is to say that they do not affect the primary sequence of the

20 resulting heliomicine or fusion peptide.

The present invention also relates to a chimeric gene (or expression cassette) comprising a coding sequence as well as heterologous regulatory elements at the 5' and 3' positions capable of

25 functioning in a host organism, in particular plant cells or plants, the coding sequence comprising at least one DNA fragment encoding heliomicine or the

"peptide-heliomicine" fusion peptide as defined above.

Host organism is understood to mean any lower or higher, mono- or pluricellular organism into which the chimeric gene according to the invention may be 5 introduced, for the production of heliomicine. It includes in particular bacteria, for example *E. coli*, yeasts, in particular of the genera *Saccharomyces* or *Kluyveromyces*, *Pichia*, fungi, in particular *Aspergillus*, a baculovirus, or preferably plant cells 10 and plants.

"Plant cell" is understood to mean according to the invention any cell derived from a plant and capable of constituting undifferentiated tissues such as calli, differentiated tissues such as embryos, plant 15 portions, plants or seeds.

"Plant" is understood to mean according to the invention any differentiated multicellular organism capable of photosynthesis, in particular monocotyledonous or dicotyledonous plants, more 20 particularly crop plants intended or otherwise as animal or human food, such as maize, wheat, rape, soyabean, rice, sugarcane, beet, tobacco, cotton and the like.

The regulatory elements necessary for the 25 expression of the DNA fragment encoding heliomicine are well known to persons skilled in the art according to the host organism. They comprise in particular promoter

sequences, transcription activators, terminator sequences, including start and stop codons. The means and methods for identifying and selecting the regulatory elements are well known to persons skilled 5 in the art.

For the transformation of microorganisms such as yeasts or bacteria, the regulatory elements are well known to persons skilled in the art, and comprise in particular promoter sequences, transcription 10 activators, transit peptides, terminator sequences and start and stop codons.

To direct the expression and the secretion of the peptide in the yeast culture medium, a DNA fragment encoding heliomicine is integrated into a shuttle 15 vector which comprises the following elements:

- markers which make it possible to select the transformants. Preferably, the *ura-3* gene is used for yeast and the gene which confers resistance to ampicilline for *E. coli*,
- 20 - a nucleic sequence allowing the replication (replication origin) of the plasmid in yeast. Preferably, the replication origin of the yeast 2i plasmid is used,
- a nucleic sequence allowing the replication 25 (replication origin) of the plasmid in *E. coli*,
- an expression cassette consisting
 - (1) of a promoter regulatory sequence. Any

promoter sequence of a gene which is naturally expressed in yeast may be used. Preferably, the promoter of the *S. cerevisiae* *Mfα1* gene is used.

(2) of a sequence encoding a signal peptide

5 (or prepeptide) in combination with a homing peptide (or propeptide). These regions are important for the correct secretion of the peptide. Preferably, the sequence encoding the pre-pro-peptide of the precursor of factor *Mfα1* is used.

10 (3) of a polyadenylation or terminator regulatory sequence. Preferably, the terminator of *S. cerevisiae* phosphoglycerate kinase (PGK) is used. In the expression cassette, the sequence encoding heliomicine is inserted downstream of the pre-pro

15 sequence and upstream of the PGK terminator.

These elements have been described in several publications including Reichhart *et al.*, 1992, Invert. Reprod. Dev., 21, pp 15-24 and Michaut *et al.*, 1996, FEBS Letters, 395, pp 6-10.

20 Preferably, yeasts of the *S. cerevisiae* species are transformed with the expression plasmid by the lithium acetate method (Ito *et al.*, 1993, J. Bacteriol., 175, pp 163-168). The transformed yeasts are selected on a selective agar medium which does not

25 contain uracil. The mass production of transformed yeasts is carried out by culturing for 24 h to 48 h in a selective liquid medium.

The transformation of microorganisms makes it possible to produce heliomicine on a larger scale. The present invention therefore also relates to a method of preparing heliomicine, comprising the steps of

5 culturing a transformed microorganism comprising a gene encoding heliomicine as defined above in an appropriate culture medium, followed by the extraction and total or partial purification of the heliomicine obtained.

Preferably, during the extraction of the
10 heliomicine produced by yeasts, the yeasts are removed by centrifugation and the culture supernatant is placed in contact with an acidic solution which may be a solution of an inorganic or organic acid, such as for example hydrochloric acid or acetic acid. The extract
15 obtained is then centrifuged at cold temperature at a speed of 4000 to 10,000 rpm at 4°C for 30 to 60 min.

The purification of heliomicine may be preceded by a step of fractionation of the supernatant obtained following the extraction step. Preferably,
20 during the fractionation step, the extract is deposited on the reversed phase in order to carry out a solid phase extraction. The washing of the molecules which are soluble in water is carried out with a dilute acidic solution and the elution of the hydrophobic
25 molecules with an appropriate eluant. Good results are obtained with trifluoroacetic acid for the washing and an eluant containing increasing quantities of

acetonitrile in dilute acidic solution.

Preferably, the purification of heliomicine is carried out in two stages: a cation-exchange HPLC followed by a reversed phase HPLC with a suitable 5 eluant which may be different from or identical to that of the preceding phase. The various steps of the purification are monitored by a test of inhibition of fungal growth in liquid medium. Preferably, the test is carried out with the fungus *Neurospora crassa*.

10 The sequence of the heliomicine produced by the transformed yeasts is analysed according to the method of sequencing by Edman degradation and by mass spectrometry. The structural characterization is carried out directly on the peptide produced, on the 15 peptide modified by reduction/alkylation as well as on fragments of the peptide. The peptide sequence and the molecular mass of the heliomicine produced were compared with those of the native heliomicine extracted from the haemolymph of *H. virescens*. The results show 20 that the two molecules have the same primary structure. The determination of the position of the disulphide bridges indicates that the arrangement of the disulphide bridges is identical in both peptides, the native peptide and the one produced by the transformed 25 microorganism.

The invention relates more particularly to the transformation of plants. As promoter regulatory

sequence in plants, it is possible to use any promoter sequence of a gene which is naturally expressed in plants, in particular a promoter of bacterial, viral or plant origin such as, for example, that of a gene for 5 the small subunit of ribulose-biscarboxylase/oxygenase (RuBisCO) or of a plant virus gene such as, for example, that of the cauliflower mosaic (19S or 35S CAMV), or a promoter which is inducible by pathogens such as the tobacco PR-1a, it being possible to use any 10 known suitable promoter. Preferably, a promoter regulatory sequence is used which promotes the overexpression of the coding sequence constitutively or induced by attack by a pathogen, such as for example that comprising at least one histone promoter as 15 described in application EP 0,507,698.

According to the invention, it is also possible to use, in combination with the promoter regulatory sequence, other regulatory sequences which are situated between the promoter and the coding 20 sequence, such as transcription activators (enhancer), such as for example the translation activator of the tobacco mosaic virus (TMV) which is described in application WO 87/07644, or of the tobacco etch virus (TEV) which is described by Carrington & Freed.

25 As polyadenylation or terminator regulatory sequence, there may be used any corresponding sequence of bacterial origin, such as for example the

Agrobacterium tumefaciens nos terminator, or alternatively of plant origin, such as for example a histone terminator as described in application EP 0,633,317.

5 According to the present invention, the chimeric gene may also be combined with a selectable marker suitable for the transformed host organism. Such selectable markers are well known to persons skilled in the art. They may include a gene for resistance to
10 antibiotics, or alternatively a gene for tolerance to herbicides for plants.

The present invention also relates to a cloning or expression vector for the transformation of a host organism containing at least one chimeric gene
15 as defined above. This vector comprises, in addition to the above chimeric gene, at least one replication origin. This vector may consist of a plasmid, a cosmid, a bacteriophage or a virus, which are transformed by the introduction of the chimeric gene according to the
20 invention. Such transformation vectors, according to the host organism to be transformed, are well known to persons skilled in the art and are widely described in the literature.

For the transformation of plant cells or of
25 plants, they may include in particular a virus which may be used for the transformation of developed plants and which contains in addition its own elements for

replication and expression. Preferably, the vector for transforming plant cells or plants according to the invention is a plasmid.

The subject of the invention is also a method 5 of transforming host organisms, in particular plant cells by integration of at least one nucleic acid fragment or a chimeric gene as defined above, which transformation may be obtained by any appropriate known means widely described in the specialized literature 10 and in particular the references cited in the present application, more particularly using the vector according to the invention.

A series of methods consists in bombarding cells, protoplasts or tissues with particles to which 15 DNA sequences are attached. Another series of methods consists in using, as means of transfer into plants, a chimeric gene inserted into an *Agrobacterium tumefaciens* Ti or *Agrobacterium rhizogenes* Ri plasmid.

Other methods may be used, such as 20 microinjection or electroporation, or alternatively direct precipitation by means of PEG.

Persons skilled in the art will make the choice of the appropriate method according to the nature of the host organism, in particular of the plant 25 cell or of the plant.

The subject of the present invention is also the host organisms, in particular plant cells or

plants, transformed and containing an effective quantity of a chimeric gene comprising a coding sequence for heliomicine defined above.

The subject of the present invention is also 5 the plants containing transformed cells, in particular the plants regenerated from the transformed cells. The regeneration is obtained by any appropriate means which depends on the nature of the species, as described for example in the above references.

10 For the methods of transforming plant cells and of regenerating plants, there may be mentioned in particular the following patents and patent applications: US 4,459,355, US 4,536,475, US 5,464,763, US 5,177,010, US 5,187,073, EP 267,159, EP 604,662, EP 15 672,752, US 4,945,050, US 5,036,006, US 5,100,792, US 5,371,014, US 5,478,744, US 5,179,022, US 5,565,346, US 5,484,956, US 5,508,468, US 5,538,877, US 5,554,798, US 5,489,520, US 5,510,318, US 5,204,253, US 5,405,765, EP 442,174, EP 486,233, EP 486,234, EP 539,563, EP 20 674,725, WO 91/02701 and WO 95/06128.

The present invention also relates to the transformed plants derived from the cultivation and/or crossing of the above regenerated plants, as well as the seeds of transformed plants.

25 The plants thus transformed are resistant to certain diseases, in particular to certain fungal or bacterial diseases. As a result, the DNA sequence

encoding heliomicine may be integrated with the main objective of producing plants resistant to the said diseases, heliomicine being effective against fungal diseases such as those caused by *Cercospora*, in

5 particular *Cercospora beticola*, *Cladosporium*, in particular *Cladosporium herbarum*, *Fusarium*, in particular *Fusarium culmorum* or *Fusarium graminearum*, or by *Phytophthora*, in particular *Phytophthora cinnamomi*.

10 The chimeric gene may also comprise, and advantageously, at least one selectable marker, such as one or more genes for tolerance to herbicides.

The DNA sequence encoding heliomicine may also be integrated as a selectable marker during the 15 transformation of plants with other sequences encoding other peptides or proteins of interest, such as for example genes for tolerance to herbicides.

Such genes for tolerance to herbicides are well known to persons skilled in the art and are in 20 particular described in patent applications EP 115,673, WO 87/04181, EP 337,899, WO 96/38567 or WO 97/04103.

Of course the transformed cells and plants according to the invention may comprise, in addition to the sequence encoding heliomicine, other heterologous 25 sequences encoding proteins of interest such as other additional peptides which are capable of conferring on the plant resistance to other diseases of bacterial or

fungal origin, and/or other sequences encoding proteins for tolerance to herbicides and/or other sequences encoding proteins for resistance to insects, such as the *Bt* proteins in particular.

5 The other sequences may be integrated by means of the same vector comprising a chimeric gene, which comprises a first sequence encoding heliomicine and at least one other sequence encoding another peptide or protein of interest.

10 They may also be integrated by means of another vector comprising at least the said other sequence, according to the customary techniques defined above.

The plants according to the invention may 15 also be obtained by crossing parents, one carrying the gene according to the invention encoding heliomicine, the other carrying a gene encoding at least one other peptide or protein of interest.

Among the sequences encoding other antifungal 20 peptides, there may be mentioned that encoding drosomycin, which is described in patent application FR 2,725,992 and by Fehlbaum *et al.* (1994), and in unpublished patent application FR 97 09115 filed on 24 July 1997, or that encoding androctonin which is 25 described in patent application FR 2,745,004 and in unpublished patent application FR 97 10362 filed on 20 August 1997.

The present invention finally relates to a method of cultivating transformed plants according to the invention, the method consisting in planting the seeds of the said transformed plants in a plot of a 5 field appropriate for cultivating the said plants, in applying to the said plot of the said field an agrochemical composition, without substantially affecting the said seeds or the said transformed plants, then in harvesting the cultivated plants when 10 they arrive at the desired maturity and optionally in separating the seeds from the harvested plants.

Agrochemical composition is understood to mean according to the invention any agrochemical composition comprising at least one active product 15 having one of the following activities: herbicide, fungicide, bactericide, virucide or insecticide.

According to a preferred embodiment of the method of cultivation according to the invention, the agrochemical composition comprises at least one active 20 product having at least one fungicidal and/or bactericidal activity, more preferably exhibiting an activity which is complementary to that of the heliomicine produced by the transformed plants according to the invention.

25 Product exhibiting an activity which is complementary to that of heliomicine is understood to mean according to the invention a product exhibiting a

complementary activity spectrum, that is to say a product which will be active against attacks by contaminants (fungi, bacteria or viruses) which are not sensitive to heliomicine, or alternatively a product 5 whose activity spectrum covers that of heliomicine, completely or in part, and whose dose for application will be substantially reduced because of the presence of the heliomicine produced by the transformed plant.

The examples below make it possible to 10 illustrate the present invention without however limiting its scope.

Example I: Isolation and characterization of heliomicine from the haemolymph collected from immunized larvae of the lepidopteron *H. virescens*

15 Example I.1: Isolation

1-1 Induction of the biological synthesis of an antifungal substance in the haemolymph of *H. virescens*

The 5th stage mature larvae of the lepidopteron *H. virescens* were immunized with the aid 20 of a needle (30 ga) previously stuck into a pellet of Gram-positive (*M. luteus*) and Gram-negative (*E. coli* 1106) bacteria which is prepared from cultures carried out in a Lauria-Bertani medium for 12 hours at 37°C. The animals thus infected were kept individually in 25 tubes containing an agar-based nutrient medium for 24 hours between 20°C and 23°C. Before collecting the haemolymph, the larvae were cooled on ice.

1-2 Preparation of the plasma

The haemolymph (about 30 μ l per larva) was collected by excision of an abdominal appendage and collected in 1.5-ml polypropylene microcentrifuge tubes 5 cooled on ice and containing aprotinin as protease inhibitor (20 μ g/ml final concentration) and phenylthiourea as melanization inhibitor (final concentration of 20 μ M). The haemolymph (2 ml) thus collected from the immunized larvae was centrifuged at 10 14,000 g for 1 min at 4°C in order to remove the haemocytes. The haemolymph, free of blood cells, was stored at -20°C up to its use.

1-3 Acidification of the plasma

After rapid thawing, the *H. virescens* plasma 15 was acidified to pH 3 with a 1% trifluoroacetic acid solution. The extraction, under acidic conditions, of the peptide was carried out for 30 min, with gentle stirring, on an ice-cold bath. The extract obtained was then centrifuged at 4°C for 30 min at 10,000 g.

20 1-4 Purification of the peptides**a) Prepurification by solid phase extraction**

A quantity of extract equivalent to 2 ml of haemolymph was deposited on a reversed-phase support, as marketed in the form of a cartridge (Sep-Pak™ C18, 25 Waters Associates), equilibrated with acidified water (0.05% TFA). The hydrophilic molecules were removed by a simple wash with acidified water. The elution of the

peptide was carried out with a 40% acetonitrile solution prepared in 0.05% TFA. The fraction eluted at 40% of acetonitrile was dried under vacuum with the aim of removing the acetonitrile and the TFA and then it 5 was reconstituted in sterile ultrapure water before being subjected to the first purification step.

b) Purification by high-performance liquid chromatography (HPLC) on a reversed-phase column

- **first step:** the fraction containing the 10 peptide was analysed by reversed-phase chromatography on an Aquapore RP-300 C₈ semipreparative column (Brownlee™, 220 × 70 mm, 300 Å), the elution was carried out using a linear gradient of acetonitrile from 2 to 60% in 0.05% TFA over 120 minutes at a 15 constant flow rate of 1.5 ml/min. The fractions were collected manually, monitoring the variation of the absorbance at 225 nm and 254 nm. The fractions collected were dried under vacuum, reconstituted with ultrapure water and analysed for their antifungal 20 activity using the test described below.

- **second step:** the antifungal fraction corresponding to the peptide was analysed on an Aquapore RP-300 C₈ reversed-phase analytical column (Brownlee™, 220 × 4.6 mm, 300 Å), using a biphasic linear gradient 25 of acetonitrile from 2% to 22% over 10 min and from 22 to 32% over 50 min in 0.05% TFA with a constant flow rate of 0.8 ml/min. The fractions were collected

manually, monitoring the variation of the absorbance at 225 nm and 254 nm. The fractions collected were dried under vacuum, reconstituted with ultrapure water and analysed for their antifungal activity under the 5 conditions described below.

- **third step:** the antifungal fraction containing the peptide was purified to homogeneity on a Narrowbore Delta-Pak™ HPIC, reversed-phase column (Waters Associates, 150 × 2.2 mm) using a biphasic linear 10 gradient of acetonitrile from 2% to 24% over 10 min and from 24 to 44% over 100 min in 0.05% TFA with a constant flow rate of 0.25 ml/min at a controlled temperature of 30°C. The fractions were collected manually, monitoring the variation of the absorbance at 15 225 nm. The fractions collected were dried under vacuum, reconstituted with filtered ultrapure water and analysed for their antifungal activity.

Example I.2: structural characterization of the peptide

2-1 Verification of purity by zonal capillary

20 electrophoresis

The purity of the antifungal peptide was verified by zonal capillary electrophoresis on a 270-HT model (PEApplied Biosystems division of Perkin Elmer).

1 nl of a 50 iM solution of purified peptide was 25 injected with the aid of a vacuum into a silica capillary (72 cm × 50 im) and the analysis was carried out in a 20 mM citrate buffer at pH 2.5. The

electrophoresis was carried out at 20 kV from the anode to the cathode for 20 min at 30°C. The migration was recorded at 200 nm.

2-2 Determination of the number of cysteines :

5 reduction and S-pyridylethylation

The number of cysteine residues was determined on the native peptide by reduction and S-pyridylethylation. 100 pmol of native peptide were reduced in 40 *il* of 0.5 M Tris-HCl buffer, pH 7.5 containing 2 mM EDTA and 6 M guanidinium chloride in the presence of 2 *il* of 2.2 M dithiothreitol. The reaction medium was placed under a nitrogen atmosphere. After incubating for 60 min in the dark, 2 *il* of freshly distilled 4-vinylpyridine were added to the reaction which was then incubated for 10 min at 45°C in the dark and under a nitrogen atmosphere. The pyridylethylated peptide was then separated from the constituents of the reaction medium by reversed-phase chromatography using a linear gradient of acetonitrile in the presence of 0.05% TFA.

2-3 Determination of the mass of the native peptide, of the S-pyridylethylated peptide and of the proteolysis fragments by MALDI-TOF (Matrix Assisted Laser Desorption Ionization-Time of Flight) mass spectrometry

The mass measurements were carried out on a Bruker Biflex MALDI-TOF mass spectrometer (Bremen, Germany) in a positive linear mode. The mass spectra

were calibrated externally with a standard mixture of peptides of known m/z, respectively 2199.5 Da, 3046.4 Da and 4890.5 Da. The various products to be analysed were deposited on a thin layer of α -cyano-4-5 hydroxycinnamic acid crystals which is obtained by rapid evaporation of a solution saturated with ethanol. After drying under a moderate vacuum, the samples were washed with a drop of 0.1% trifluoroacetic acid before being introduced into the mass spectrum.

10 2-4 Sequencing by Edman degradation

The automated sequencing by Edman degradation of the native peptide, of the S-pyridylethylated peptide and of the various fragments obtained after the various proteolytic cleavages and the detection of the 15 phenylthiohydantoin derivatives were carried out on an ABI473A sequencer (PEApplied Biosystems division of Perkin Elmer).

2-5 Proteolytic cleavages

- Confirmation of the peptide sequence in the C-terminal region

200 pmol of reduced and S-pyridylethylated peptide were incubated in the presence of 5 pmol of endoproteinase-Lys-C (Acromobacter protease I, specific cleavage of the lysine residues on the C-terminal side, 25 Takara, Otsu) according to the conditions recommended by the supplier (10 mM Tris-HCl, pH 9, in the presence of 0.01% Tween 20). After stopping the reaction with 1%

TFA, the peptide fragments were separated by reversed-phase HPLC on a Narrowbore Delta-Pak™ HPIC₁₈ type column (Waters Associates 150 × 2 mm) in a linear gradient of acetonitrile from 2 to 60% over 80 min in 0.05% TFA

5 with a flow rate of 0.2 ml/min and a constant temperature of 37°C. The fragments obtained were analysed by MALDI-TOF mass spectrometry and the peptide corresponding to the C-terminal fragment was sequenced by Edman degradation.

10 - **Determination of the arrangement of the disulphide bridges by proteolysis with thermolysin**

The native peptide (8 µg) was incubated for 1 hour in the presence of 4 µg of thermolysin (Boehringer Mannheim, thermolysin/peptide ratio, 1/2 by weight :

15 weight) at 37°C in 0.1 M MES (N-ethylmorpholine) buffer at pH 7 in the presence of 2 mM CaCl₂. The reaction was stopped by addition of formic acid and the reaction products were immediately separated by reversed-phase chromatography on a Narrowbore Delta-Pak™ HPIC₁₈ column (Waters Associates, 150 × 2.2 mm) in a linear gradient of acetonitrile from 2 to 50% over 100 min in 0.05% TFA

20 at the flow rate of 0.2 ml/min at 30°C preceded by an isocratic step at 2% acetonitrile over 10 min. The fragments obtained were analysed by MALDI-TOF mass spectrometry and sequenced by Edman degradation.

25

Example II: Expression of heliomicine in the yeast *Saccharomyces cerevisiae*

All the techniques used below are standard laboratory techniques. The detailed protocols for these techniques have been described in particular in Ausubel et al.

5 **Example II-1: Assembling of the synthetic gene**

Assembling was carried out using 6 synthetic oligonucleotides encoding the 44 amino acids of heliomicine preceded by the 5 C-terminal amino acids of the pre-pro sequence of factor α1 (Mfa1) of the yeast.

10 The oligonucleotides represented in Figure 1 were chosen taking into account the preferential codons used by *S. cerevisiae*.

The assembling took place in several steps:

- oligonucleotides 2 to 5 were phosphorylated
15 at their 5' ends by the action of polynucleotide kinase (New England Biolabs);

- oligonucleotides 1 to 6 were mixed, heated to 100°C and hybridized by slowly reducing the temperature to 25°C over 3 hours;

20 - the hybridized oligonucleotides were subjected to a treatment with T4 bacteriophage ligase (New England Biolabs) for 15 hours at 15°C;

- the DNA unit resulting from the hybridization of the oligonucleotides which is
25 represented in Figure 1, flanked by the *Hin*DIII and *Bgl*II restriction sites, was inserted into the plasmid pBluescript SK+ (Stratagene) digested with the enzymes

HinDIII and BamHI (BglII and BamHI are compatible). The ligation mixture was then used to transform competent *E. coli* DH5 α cells (Stratagene). Several clones were analysed and sequenced. One of these clones which had

5 the desired sequence was called pSEA1.

Example II-2: Construction of the vector pSEA2 which allows the secretion of the heliomicine synthesized

The HinDIII-SalI DNA fragment of the vector pSEA1, carrying the sequence encoding heliomicine as

10 well as the SphI-HinDIII fragment of the vector M13JM132 (Michaut et al., 1985, FEBS Letters, 395, pp 6-10) were inserted between the SphI and SalI sites of the plasmid pTG4812 (Michaut et al., 1996, FEBS Letters, 395, pp 6-10). The SphI-HinDIII fragment of

15 the vector M13JM132 contains the sequence of the promoter of the MF α 1 gene of the yeast as well as the sequence encoding the pre-pro region of factor MF α 1. In the resulting plasmid pSEA2, the synthetic gene for heliomicine therefore finds itself inserted between the

20 pre-pro sequences of factor Mf α 1 and the transcription terminator; this construct should therefore ensure the maturation and the secretion of heliomicine.

Example II-3: Transformation of a strain of *S. cerevisiae* with the DNA of the plasmid pSEA2 and

25 **analysis of the transformants**

The yeast strain TGY 48.1 (MAT α , ura3-D5, his, pral, prb1, prc1, cps1; Reichhart et al., 1992,

Invert. Reprod. Dev. 21, pp 15-24) was transformed with the plasmid pSEA2. The transformants were selected at 29°C on a selective YNBG medium (0.67% yeast nitrogen base, 2% glucose), supplemented with 0.5% of casamino acids and containing no uracil. After transformation, several yeast clones, selected for the ura+ character, were cultured for 48 h at 29°C in 50 ml of selective medium. After centrifugation (4000 g, 30 min, 4°C), the supernatant was acidified to pH 3.5 with acetic acid, 10 before being deposited on a Sep-Pak™ C₁₈ cartridge (Waters Associates) equilibrated with acidified water (0.05% TFA). The various proteins bound to the cartridge were eluted with solutions of 0.05% TFA containing increasing percentages of acetonitrile.

15 The 40% fraction, exhibiting an antifungal activity, was analysed by HPLC on an Aquapore RP-300 C₈ reversed-phase analytical column (Brownlee™, 220 × 4.6 mm, 300 Å), using a linear gradient of acetonitrile from 2% to 40% over 80 min in 0.05% TFA 20 with a constant flow rate of 0.8 ml/min. The fractions were collected manually by monitoring the variation in absorbance at 225 nm and 254 nm. The fractions collected were dried under vacuum, reconstituted with ultrapure water and analysed for their antifungal 25 activity under the conditions described in Example III. The structural characterization of the peptide was carried out as described in Example I.2.

Example II-4: Production of recombinant heliomicine on a semipreparative scale

One of the clones of transformed yeast expressing heliomicine was cultured at 29°C for 24 h in 5 100 ml of selective medium. This procedure was then used to inoculate 4 l of selective medium and the culture was carried out for 48 h at 29°C. The yeasts were removed by centrifugation (4000 g, 30 min, 4°C). The supernatant was acidified to pH 3.5 with acetic 10 acid, subjected to a second centrifugation (4000 g, 30 min, 4°C) before being deposited on a C₁₈ preparative reversed-phase open column (Waters Associates), 125 Å, 6 g of phase per 500 ml of supernatant) equilibrated with acidified water (0.05% TFA). The hydrophilic 15 molecules were removed by a wash with acidified water followed by a wash with a 15% solution of acetonitrile prepared in 0.05% TFA. The elution of the peptide was carried out using a 40% acetonitrile solution prepared in 0.05% TFA. The fraction eluted at 40% acetonitrile 20 was lyophilized and then reconstituted in sterile ultrapure water before being subjected to the first purification step.

- first step of purification by HPLC: the purified fraction containing heliomicine was 25 reconstituted in a 25 mM ammonium acetate solution, pH 3.4. This sample was injected into an Aquapore Cation Exchange preparative cation-exchange column (Brownlee™,

250 x 10 mm), using a linear gradient of NaCl from 0% to 100% over 90 min in 25 mM ammonium acetate, pH 3.4 with a constant flow rate of 2 ml/min. The fractions collected were dried under vacuum, reconstituted with 5 ultrapure water and analysed for their antifungal activity under the conditions described below.

- second step of purification by HPLC: the heliomicine was purified to homogeneity by chromatography on an Aquapore RP-300 C₈ semipreparative 10 reversed-phase column (Brownlee™, 220 x 7 mm, 300 Å), using a linear gradient of acetonitrile from 2% to 40% over 80 min in 0.05% TFA with a constant flow rate of 2 ml/min.

Example III: Test of activity in vitro: measurement of 15 the antifungal activity by microspectrophotometry

This methodology was used to test for the antifungal molecules during the various purification steps, for the determination of the activity spectrum of the peptide and for the determination of the minimum 20 inhibitory concentration (MIC) at which the peptide was active. The MIC was expressed as the concentration range [a] - [b] where [a] was the minimum concentration where the start of growth is observed and [b] the concentration for which no growth was observed.

25 Examples of the specific activity of heliomicine, against filamentous fungi and yeasts, are given in Tables 1 and 2.

Example III-1: Test for detection of activity against filamentous fungi

The antifungal activity was detected by a test for inhibition of growth in a liquid medium. The 5 spores of the fungi to be tested were suspended in a culture medium of the "potato-glucose" type.

Preferably, 12 g of Potato Dextrose Broth medium (Difco) were used per 1 l of demineralized water. Two antibiotics were added to the culture medium:

10 tetracycline (final concentration of 10 μ g/ml) and cefotaxime (100 μ g/ml). 10 μ l of each fraction to be analysed are deposited in microtitre plates in the presence of 90 μ l of culture medium containing the spores (at a final concentration of 10⁴ spores/ml). The 15 incubation was carried out in a humid chamber at 30°C for 48 hours. Fungal growth was observed under a light microscope after 24 h and quantified after 48 hours by measuring the absorbance at 600 nm with the aid of a spectrophotometric microtitre plate reader.

20 - filamentous fungi tested: *Aspergillus fumigatus* (gift from Dr H. Koenig, Hôpital civil, Strasbourg); *Nectria haemotococca*, *Fusarium culmorum*, *Trichoderma viride* (fungus culture collection of the Université Catholique of Leuven, Belgium); *Neurospora crassa*, *Fusarium oxysporum*, (fungus culture collection of Société Clause, Paris).

The results of the test of heliomicine

activity against filamentous fungi are presented in Table 1 below.

Table 1: activity of heliomicine against

Fungi	filamentous fungi	MIC of heliomicine (iM)
<i>Neurospora crassa</i>		0.1-0.2
<i>Fusarium culmorum</i>		0.2-0.4
<i>Fusarium oxysporum</i>		1.5-3
<i>Nectria haematococca</i>		0.4-0.8
<i>Trichoderma viride</i>		1.5-3
<i>Aspergillus fumigatus</i>		6-12.5

5

Example III-2: Test for detection of activity against yeasts

The various yeast strains were incubated in a "Sabouraud" type culture medium and incubated at 30°C
 10 for 24 h with gentle stirring. The test sample (10 il) was deposited in microtitre plate wells to which there were added 90 il of a dilute yeast culture whose density was adjusted to OD 600 = 0.001. Growth was evaluated by measuring the absorbance at 600 nm with
 15 the aid of a spectrophotometric microtitre plate reader.

- yeasts tested: *Candida albicans*,
C. glabrata, *C. tropicalis*, *C. krusei*, *C. inconspicua*,
Cryptococcus neoformans, *Cryp. albidus*, *Saccharomyces*
 20 *cerevisiae* (gift from Dr H. Koenig, Hôpital civil,

Strasbourg).

The results of the test of heliomicine activity against yeasts are presented in Table 2 below.

Table 2: activity of heliomicine against yeasts

Yeasts	MIC of heliomicine (iM)
Candida albicans	2.5-5
Candida tropicalis	2.5-5
Candida krusei	10-20
Candida inconspicua	5-10
Cryptococcus neoformans	2.5-5
Cryptococcus albidus	5-10

5

These results show the excellent antifungal activity of the peptide according to the invention.

Example IV: Preparation of a transformed plant comprising a gene encoding heliomicine

10 This example describes the preparation of the sequence encoding heliomicine for its expression in a plant cell, of the chimeric gene, of the integrating vector and of the transformed plants. Figures 2 to 6 in the annexe describe the schematic structures of some 15 plasmids prepared for the construction of the chimeric genes. In these figures, the various restriction sites are marked in *italics*.

20 All the techniques used below are standard laboratory techniques. The detailed protocols for these techniques are in particular described in Ausubel et

al.

Example IV-1: Construction of the chimeric genes for the transformation of plants

pRPA-MD-P: Creation of a plasmid containing the signal 5 peptide of the tobacco PR-1 α gene

The two complementary synthetic oligonucleotides Oligo 7 and Oligo 8 below are hybridized at 65°C for 5 minutes and by slow reduction of the temperature to 30°C over 30'.

Oligo 7: 5' GCGTCGACGC GATGGGTTTC GTGCTTTCT CTCAGCTTCC
10 ATCTTCCCTT CTTGTGTCTA CTCTTCTTCT TTTCC 3'
(*SEK 50, N. 5*)

Oligo 8: 5' TCGCCGGCAC GGCAAGAGTA AGAGATCACA AGGAAAAGAA
GAAGAGTAGA CACAAGAAGG AAAGATGGAA GC 3'
(*SEK 50 N. 6*)

After hybridization between Oligo 7 and Oligo 8, the DNA remaining single-stranded serves as template for the Klenow fragment of *E. coli* polymerase I (under the standard conditions recommended by the manufacturer (New England Biolabs)) for the creation of the double-stranded oligonucleotide starting from the 3' end of 20 each oligo. The double-stranded oligonucleotide obtained is then digested with the restriction enzymes SacII and NaeI and cloned into the plasmid pBS II SK(-) (Stratagene) digested with the same restriction enzymes. A clone is then obtained which comprises the 25 region encoding the signal peptide of the tobacco PR-1 α

gene (SEQ ID NO 4).

pRPA-PS-PR1 α -helio: Creation of a sequence encoding heliomicine fused with the PR-1 α signal peptide with no untranscribed region in 3'

5 The two synthetic oligonucleotides complementary to Oligo 9 and Oligo 10 sequences according to the operating conditions described for pRPA-MD-P.

Oligo 9: 5' GATAAGCTTA TCGGTTCTTG CGTGTGGGT GCTGTGAAC
ACACTTCCGA TTGCAACGGT GAGTGCAAGA GGAGGGTTA 3'
(SEQ I N. 7)

Oligo 10: 5' CCGGATCCGT CGACACGGTC GCCTCGCCGA GCTCTCAAGT
CTCGCACCAG CAGTTCACGT TAGCGAAGGA ACCGCAGTGA
CCACCCCTTGT AACCCCTCCT CTTGCACTC 3'
(SEQ I N. 8)

10 After hybridization between Oligo 9 and Oligo 10, the DNA remaining single-stranded serves as template for the Klenow fragment of *E. coli* polymerase I (under the standard conditions recommended by the manufacturer (New England Biolabs)) for the creation of the double-stranded oligonucleotide starting from the 3' end of each oligo. This double-stranded oligonucleotide containing the coding portion of 15 heliomicine (SEQ ID NO 2) is then cloned directly into the plasmid pRPA-MD-P which has been digested with the restriction enzyme NaeI. The correct orientation of the clone obtained is checked by sequencing. A clone is 20 then obtained which comprises the region encoding the PR-1 α -heliomicine fusion protein situated between the 25

NcoI restriction sites at the N-terminal end and the ScaI, SacII and BamHI restriction sites at the C-terminal end (SEQ ID NO 3).

pRPA-RD-239: Creation of a vector for expression in
5 **plants comprising the sequence encoding the PR-1 α -heliomicine fusion protein**

The plasmid pRTL-2 GUS, derived from the plasmid pUC-19, was obtained from Dr Jim Carrington (Texas A&M University, not described). This plasmid, 10 whose schematic structure is represented in Figure 2, contains the duplicated CaMV 35S promoter isolated from the cauliflower mosaic virus (CaMV 2x35S promoter; Odell *et al.*, 1985) which directs the expression of an RNA containing the tobacco etch virus 5' untranslated 15 sequence (TEV 5' UTR; Carrington & Freed, 1990), the *E. coli* β -glucuronidase gene (GUS Jefferson *et al.*, 1987) followed by the CaMV 35S RNA polyadenylation site (CaMV polyA; Odell *et al.*, 1985).

The plasmid pRTL-2 GUS is digested with the 20 restriction enzymes NcoI and BamHI and the large DNA fragment is purified. The plasmid pRPA-PS-PR1 α -helio is digested with the restriction enzymes NcoI and BamHI and the small DNA fragment containing the region encoding the PR-1 α -heliomicine fusion protein is 25 purified. The two purified DNA fragments are then ligated together into a cassette for expression in plants which synthesizes a PR-1 α -heliomicine fusion

protein. The schematic structure of this expression cassette is represented in Figure 3. "PR-1 α -heliomicine" represents the coding region for the PR-1 α -heliomicine fusion protein of pRPA-RD-239. The 5 heliomicine is transported to the extracellular matrix of the plant by the action of the PR-1 α signal peptide.

pRPA-RD-195: Creation of a plasmid containing a modified multiple cloning site

The plasmid pRPA-RD-195 is a plasmid derived 10 from pUC-19 which contains a modified multiple cloning site. The complementary synthetic oligonucleotides Oligo 11 and Oligo 12 below are hybridized and made double-stranded according to the procedure described for pRPA-MD-P.

Oligo 11: 5' AGGGCCCCCT AGGGTTAAA CGGCCAGTCA GGCGAATTG
15 GAGCTCGGTA CCCGGGGATC CTCTAGAGTC GACCTGCAGG
CATGC 3' (SEQ. 9)
Oligo 12: 5' CCCTGAACCA GGCTCGAGGG CGCGCCTTAA TTAAAAGCTT
GCATGCCTGC AGGTCGACTC TAGAGG 3'
(SEQ. 10)

The double-stranded oligonucleotide obtained is then ligated into pUC-19 which has been previously 20 digested with the restriction enzymes EcoRI and HindIII and made blunt-ended using the Klenow fragment of *E. coli* DNA polymerase I. A vector is obtained which contains multiple cloning sites in order to facilitate the introduction of the cassettes for expression in an 25 *Agrobacterium tumefaciens* vector plasmid. The schematic

structure of this multiple cloning site is represented in Figure 4.

pRPA-RD-240: Introduction of the cassette for expression of PR-1 α -heliomicine from pRPA-RD-239 into

5 **pRPA-RD-195**

The plasmid pRPA-RD-239 is digested with the restriction enzyme PstII. The DNA fragment containing the cassette for expression of PR-1 α -heliomicine is purified. The purified fragment is then ligated into 10 pRPA-RD-195 which has been previously digested with the restriction enzyme PstII and dephosphorylated with calf intestinal phosphatase.

pRPA-RD-174: Plasmid derived from pRPA-BL-150A (EP 0,508,909) containing the gene for tolerance to 15 bromoxynil of pRPA-BL-237 (EP 0,508,909)

The gene for tolerance to bromoxynil is isolated from pRPA-BL-237 by gene amplification by PCR. The fragment obtained is blunt-ended and is cloned into the EcoRI site of pRPA-BL-150A which has been made 20 blunt-ended by the action of Klenow polymerase under standard conditions. An *Agrobacterium tumefaciens* vector is obtained which contains the gene for tolerance to bromoxynil near its right border, a gene for tolerance to kanamycin near its left border and a 25 multiple cloning site between these two genes.

The schematic structure of pRPA-RD-174 is represented in Figure 5. In this figure, "nos"

represents the *Agrobacterium tumefaciens* nopaline synthase polyadenylation site (Bevan et al., 1983), "NOS pro" represents the *Agrobacterium tumefaciens* nopaline synthase promoter (Bevan et al., 1983), "NPT 5 II" represents the *E. coli* Tn5 transposon neomycin phosphotransferase gene (Rothstein et al., 1981), "35S pro" represents the 35S promoter isolated from the cauliflower mosaic virus (Odell et al., 1985), "BRX" represents the nitrilase gene isolated from *K. ozaenae* (Stalker et al., 1988), "RB" and "LB" represent the right and left borders respectively of the sequence of an *Agrobacterium tumefaciens* Ti plasmid.

pRPA-RD-184: Addition of a new unique restriction site into pRPA-RD-174

15 The complementary synthetic oligonucleotides Oligo 13 and Oligo 14 below are hybridized and made blunt-ended according to the procedure described for pRPA-MD-P.

Oligo 13: 5' CCGGCCAGTC AGGCCACACT TAATTAAGTT TAAACGCGGC
CCCGGCGCGC CTAGGTGTGT GCTCGAGGGC CCAACCTCAG
TACCTGGTTC AGG 3' (5'9.1A N.11)

Oligo 14: 5' CCGGCCTGAA CCAGGTACTG AGGTTGGGCC CTCGAGCACA
CACCTAGGCG CGCCGGGGCC GCGTTAAC TTAATTAAGT
20 GTGGCCTGAC TGG 3' (5'9.1A N.12)

The hybridized double-stranded oligonucleotide (95 base pairs) is purified after separation on an agarose gel (3% Nusieve, FMC). The

plasmid pRPA-RD-174 is digested with the restriction enzyme XmaI, and the large DNA fragment is purified. The two DNA fragments obtained are then ligated.

A plasmid derived from pRPA-RD-174 is obtained which comprises other restriction sites 5 between the gene for tolerance to bromoxynil and the selectable marker kanamycin gene.

The schematic structure of the plasmid pRPA-RD-184 is represented in Figure 6 where the terms 10 "nos", "NPT-II", "NOS pro", 35S pro", "BRX gene", "RB" and "LB" have the same meaning as in Figure 5.

pRPA-RD-241: Creation of an *Agrobacterium tumefaciens* vector containing the construct of the gene encoding heliomicine directed towards the extracellular matrix

15 The plasmid pRPA-RD-240 is digested with the restriction enzymes SfiII and AscI and the DNA fragment containing the PR-1 α -heliomicine gene is purified. The plasmid pRPA-RD-184 is digested with the same restriction enzymes. The DNA fragment containing the 20 cassette for expression of PR-1 α -heliomicine is then ligated into pRPA-RD-184. An *Agrobacterium tumefaciens* vector is thus obtained which contains the sequence encoding the PR-1 α -heliomicine fusion protein which leads to the expression of heliomicine in the 25 extracellular matrix of the plant.

Example IV-2: Creation of an expression cassette CsVMV promoter - PG1 signal peptide - heliomicine - Nos

terminator

pRPA-NP4: Creation of a plasmid containing the signal peptide of the maize polygalacturonase PG1 gene (Genbank, accession No. X57627)

5 The two partially complementary synthetic oligonucleotides Oligo 13 and Oligo 14 below are hybridized at 65°C for 5 minutes and then by slowly reducing the temperature to 30°C over 30 minutes.

Oligo 15: 5' GGTCTAGAAT GGCCTGCACC AACAAACGCCA TGAGGGCCCT
CTTCCTCCTC 3' (SEQ. ID NO. 13)

Oligo 16: 5' CCGAATTCTGG CGCCGTGCAC GATGCAGAAG AGCACGAGGA
GGAAGAGGGC 3' (SEQ. ID NO. 14)

10

After hybridization between Oligo 13 and Oligo 14, the DNA remaining single-stranded serves as template for the Klenow fragment of *E. coli* polymerase I (under the standard conditions recommended by the manufacturer (New England Biolabs)) for the creation of the double-stranded oligonucleotide starting from the 3' end of each oligo. The double-stranded oligonucleotide obtained is then digested with the restriction enzymes XbaI and EcoRI and then cloned into the plasmid pBS II SK(-) (Stratagene) digested with the same restriction enzymes. A clone is then obtained which contains the region encoding the 22 amino acids of the signal peptide of the PG1 gene, and which may be fused with the reading frame of other proteins at the level of the SfoI site (SEQ ID NO 15).

pRPA-NP5: Creation of a sequence encoding heliomicine fused with the signal peptide of the PG1 gene

The region encoding heliomicine was amplified by PCR from the clone pRPA-PS-PR1 α -helio (SEQ ID NO 3) 5 with the thermostable Pfu enzyme (Stratagene) according to the standard conditions recommended by the manufacturer. The synthetic oligonucleotides used for this amplification are:

Oligo 17: 5' GATAAGCTTA TCGGTTCCCTG CGTG 3' (SEQ ID NO.16)
10 Oligo 18: 5' GGCTCGAGTC AAGTCTCGCA CCAGCAGTTC AC 3' (SEQ ID NO.17)

The PCR product was digested with the restriction enzyme XhoI and cloned into the vector pRPA-NP4 digested with the restriction enzymes SfoI and XhoI. The resulting clone therefore comprises the 15 region encoding the signal peptide of the PG1 gene fused in the same reading frame with the sequence encoding heliomicine (SEQ ID NO 18).

pRPA-NP6: Creation of a cassette for expression of heliomicine in a transformation vector

20 The expression and transformation vector pILTAB 357 is derived from the binary vector pBin19. It contains the CsVMV promoter (Verdaguer et al. 1996, Plant Mol. Biol. 31, 1129-1139) followed by a multiple cloning site and the nopaline synthase Nos 25 transcription terminator (Figure X+1). The sequence of this fragment is indicated (SEQ ID NO 19).

The heliomicine expression vector was

obtained by insertion of the *Xba*I-*Kpn*I restriction fragment of the vector pRPA-NP5 containing the PG1 signal peptide-heliomicine fusion into the vector PILTAB 357 digested with these same enzymes. The 5 resulting clone therefore contains the expression cassette CsVMV promoter-PG1 signal peptide-heliomicine-Nos terminator (SEQ ID NO 20).

Example IV-3: Preparation of transformed tobacco

3.1 - Transformation

10 The vectors pRPA-RD-241 and pRPA-NP6 are introduced into the *Agrobacterium tumefaciens* EHA101 or EHA105 strain (Hood et al., 1987) carrying the cosmid pTVK291 (Komari et al., 1986). The transformation technique is based on the procedure of Horsh et al. 15 (1985).

3.2- Regeneration

The regeneration of the PBD6 tobacco (origin SEITA France) from foliar explants is carried out on a Murashige and Skoog (MS) basic medium comprising 30 g/l 20 of sucrose as well as 200 μ g/ml of kanamycin. The foliar explants are collected from plants cultivated in a greenhouse or *in vitro* and regenerated according to the foliar disc technique (Horsh et al., 1985) in three successive stages: the first comprises the induction of 25 shoots on a medium supplemented with 30 g/l of sucrose containing 0.05 mg/l of naphthylacetic acid (ANA) and 2 mg/l of benzylaminopurine (BAP) for 15 days. The

shoots formed during this stage are then developed for 10 days by culturing on an MS medium supplemented with 30 g/l of sucrose but containing no hormone. Next, developed shoots are collected and they are cultivated 5 on an MS rooting medium containing half the content of salt, vitamins and sugar and containing no hormone. After about 15 days, the rooted shoots are transferred into soil.

3.3- Analysis of the expression of heliomicine in 10 transgenic tobacco

a) Production of specific polyclonal antibodies

Polyclonal antibodies were obtained by immunizing a rabbit with native heliomicine according 15 to the usual procedures of the Centre de Bioexpérimentation VALBEX (IUT A - Lyon I). The serum obtained (15 ml) was then immunopurified on Sepharose 4B column (Pharmacia; ref 17-0430-01) coupled to heliomicine so as to specifically select the 20 immunoglobulins which recognize this peptide. These antibodies were finally eluted in 6 ml of glycine (200 mM; pH 3), neutralized with 1 M Tris pH 9.5, dialysed with 0.5× PBS, and stored frozen at -20°C up to the time of use.

25 b) Immunodetection of heliomicine in transgenic tobacco

Analysis of the expression of heliomicine was

conducted on 8 transgenic plants for the construct pRPA-NP6, as well as on a wild-type control. Well-developed leaves of tobacco in a greenhouse were finely ground at the temperature of liquid nitrogen, and the 5 proteins extracted for 1 h at 4°C in 50 mM Tris-HCl buffer, 1% PVP25, 0.05% Triton X100, pH 7.5 in an amount of 4 ml of buffer per gram of fresh weight. After centrifugation, the concentration of protein in the supernatant was determined by the Bradford method.

10 Five micrograms of protein of each of the 9 extracts were deposited on nitrocellulose membrane in a "slot-blot" format, as well as a quantity of 50 ng of pure heliomicine which serves as positive control. The membrane was incubated for 1 h in 1% blocking buffer 15 (Boehringer; ref 1 921 673) in TBS, and then incubated overnight at 4°C with immunopurified antibodies directed against heliomicine, diluted, 1/2000 in TBS buffer with 0.05% Tween 20. After washing (TBS, 0.1 Tween 20 and 0.5% of blocking buffer), the membrane was 20 incubated for 1 h at room temperature (TBS with 0.5% blocking buffer) with a goat antibody (diluted 1/50 000) directed specifically against rabbit immunoglobulins and coupled to alkaline phosphatase (SIGMA A-3687). After washing (TBS, 0.1% Tween 20), the 25 detection is made by adding a phosphatase substrate (BioRad; ref 170-5012), and the revealing is obtained by radiography of the luminescent product on Amersham

film (ECL).

The result of this experiment shows that 4 transgenic tobacco plants strongly express heliomicine. The signal in the other transgenic plants is weak or 5 not significant compared with the wild-type control. The signal observed for the best plant is at the level of the positive control (50 ng of heliomicine), which indicates that in this plant, heliomicine represents by weight about 1% of the total proteins.

10 **Example V-1: emulsifiable concentrates**

Example EC1:

-active substance	400 g/l
-alkali metal dodecylbenzenesulphonate	24 g/l
-oxyethylated nonylphenol containing 10 16 g/l molecules of ethylene oxide	
-cyclohexanone	200 g/l
-aromatic solvent	qs 1 litre

Example EC2:

-active substance	250 g
-epoxidized vegetable oil	25 g
-mixture of alkylarylsulphonate and polyglycol ether and fatty alcohols	100 g
-dimethylformamide	50 g
-xylene	575 g

Example V-2: flowableExample F 1:

-active substance	500 g
-polyethoxylated tristyrylphenol phosphate	50 g/l
-polyethoxylated alkylphenol	50 g
-sodium carboxylate	20 g
-ethylene glycol	50 g
-organopolysiloxane oil (antifoam)	1 g
-polysaccharide	1.5 g
-water	316.5 g

Example V-3: wettable powders (or spraying powders):Example WP 1

-active substance	50%
-ethoxylated fatty alcohol (wetting agent)	2.5%
-ethoxylated phenylethylphenol (dispersing agent)	5%
-chalk (inert carrier)	42.5%

Example WP 2:

-active substance	10%
-C13, branched type oxo synthetic alcohol ethoxylated with 8 to 10 ethylene oxide (wetting agnt)	0.75%
-neutral calcium lignosulphonate (dispersing agent)	12%
-calcium carbonate (inert filler)	qs 100%

Example WP 3:

-active substance	75%
-wetting agent	1.50%
-dispersing agent	8%
-calcium carbonate (inert filler)	qs 100%

Example WP 4:

-active substance	90%
-ethoxylated fatty alcohol (wetting agent)	4%
-ethoxylated phenylethylphenol (dispersing agent)	6%

Example WP 5:

-active substance	50%
-mixture of anionic and nonionic surfactants (wetting agent)	2.5%
-sodium lignosulphonate (dispersing agent)	5%
-kaolinic clay (inert carrier)	42.5%

Example V-4: dispersible granulesExample DG 1

5 90% by weight of active substance and 10% of pearl urea are mixed in a mixer. The mixture is then ground in a toothed roll grinder. A powder is obtained which is wetted with about 8% by weight of water. The wet powder is extruded in a perforated roll extruder.

10 Granules are obtained which are dried and then crushed and sieved so as to retain respectively only the

granules having a size of between 150 and 2000 microns.

Example DG2:

The following constituents are mixed in a mixer:

-active substance	75%
-wetting agent (sodium alkyl naphthalene sulphonate)	2%
-dispersing agent (sodium polynaphthalene sulphonate)	8%
-inert filler insoluble in water (kaolin)	15%

This mixture is granulated on a fluidized bed, in the presence of water, and then dried, crushed 5 and sieved so as to obtain granules having a size of between 0.15 and 0.80 mm.

Example V-5: pharmaceutical compositions

Example A: tablets

Tablets containing 50 mg doses of active 10 peptide having the following composition are prepared according to the usual technique:

- peptide heliomicine M1	50 mg
- starch	60 mg
- lactose	50 mg
- magnesium stearate	2 mg

Example B: injectable solution

An injectable solution containing 20 mg of 15 active peptide having the following composition is

prepared:

- peptide heliomicine M 2	22.4 mg
- distilled water	qs 2 cm ³

Example VI. Stability of the activity of heliomicine

5 The stability of an antimicrobial peptide towards plant proteases is an essential factor for obtaining a good level of expression and therefore of resistance to phytopathogens in transgenic plants. This stability is for example a critical point for an insect 10 antimicrobial peptide such as cecropin B (Owens and Heutte, 1997, MPMI vol 10, No. 4, pp 525-528). We examined the stability of heliomicine and of its activity on a test phytopathogen (*Botrytis cinerea*) after incubation with crude extracts of 8 major crop 15 plants (maize, wheat, barley, rape, soyabean, sunflower, tomato and tobacco).

 The leaves of these 8 species were ground at low temperature (liquid nitrogen) in a mortar, and then the powder was resuspended in the same volume of water.

20 After centrifugation (10,000 g for 30 minutes), the supernatant was recovered and the protein concentration determined. This concentration was adjusted for the 8 extracts to 1 mg/ml by dilution with water and then these extracts were filtered sterilely (0.2 microns). 25 One hundred microlitres of each extract (as well as a

control with only water) were then added to 50 microlitres of a solution of heliomicine (containing 15 micrograms, as well as a control without peptide) in water. These mixtures were incubated at 30°C, one aliquot of 20 microlitres collected after 0 h, 1 h, 2 h, 4 h and 20 h and immediately frozen up to the test.

The test of antifungal activity was carried out at 25°C in microplates by adding each aliquot to 80 microlitres of a fresh suspension of *Botrytis cinerea* spores (10,000 spores/ml in a solution of Potato Dextrose Broth (Difco, 12 g/l)). Visual reading of the results after 12 h and 24 h shows that there is no significant loss of antifungal activity of heliomicine even for the sample incubated for 20 h at 30°C, linked to the exposure of a crude extract of maize, wheat, barley, rape, soyabean, sunflower, tomato or tobacco. This result indicates a very high stability of heliomicine to plant proteases, and that the antifungal activity tested on *Botrytis cinerea* is not affected by the presence of crude plant extracts.

**Example VII: Production of various heliomicine mutants:
single, double, triple and quadruple mutants**

The mutants below are prepared according to the method described in Example II by replacing some of the oligonucleotides 1 to 6 with other oligonucleotides

chosen in order to introduce the mutations.

- **heliomicine R48**: replacement of the amino acid Glu48 of the sequence ID NO: 1 with a basic amino acid, in particular an arginine (Arg48). By analogy 5 with the sequence encoding the heliomicine having the sequence: SEQ ID NO: 1, the codon GAA encoding Glu is replaced by the codon AGA encoding Arg. The oligonucleotides 19 and 20 are used as a replacement for the oligonucleotides 5 and 6 of Example II.

10 Oligo 19: 5' GATCCTTCGC TAACGTTAAC TGTTGGTGTAA
GAACCTGATA GG 3' (SEG 1A NO 27)

Oligo 20: 5' TCGACCTATC AGGTTCTACA CCAACAGTTA
ACGTTAGCGA AG 3' (SEG 1B NO 28)

- **heliomicine L28L29**: replacement of two 15 basic amino acids Lys and Arg at position 28 and 29 of the sequence ID NO: 1 with two hydrophobic amino acids, in particular two leucine amino acids (Leu28 and 28). By analogy with the sequence encoding the heliomicine having the sequence: SEQ ID NO:1, the part AAG-CGC 20 encoding the peptide residue Lys-Arg is replaced by the sequence TTG-TTG encoding the peptide residue Leu-Leu. The oligonucleotides 21 and 22 are used as a replacement for the oligonucleotides 3 and 4 of Example II.

25 Oligo 21: 5' CTAGTGACTG CAACGGCGAG TGCTTGTTGC GC 3' (SEG 1B NO 29)

Oligo 22: 5' GCAACAAGCA CTCGCCGTTG CAGTCA 3' (SEG 1B NO 30)

- **heliomicine L28L29R48**: replacement of the

two basic amino acids Lys and Arg at position 28 and 29 of the sequence ID NO: 1 by two leucine amino acid residues and replacement of the amino acid Glu48 of the sequence ID NO: 1 by the amino acid arginine (Arg48).

5 The oligonucleotides 19 to 22 are used as a replacement for the oligonucleotides 3 to 6 according to Example II.

- heliomicine A24A25: replacement of the two amino acids Asn24 and Gly25 of the sequence ID NO: 1

10 two alanine amino acids (Ala24 and Ala25). By analogy with the sequence encoding the heliomicine of the sequence ID NO: 1, the part AAC-GGC encoding the peptide residue Asn-Gly is replaced by the sequence GCT-GCT encoding Ala-Ala. The oligonucleotides 23 and 15 24 are used as a replacement for the oligonucleotides 3 and 4 of Example II.

Oligo 23: 5' CTAGTGACTG CGCTGCTGAG TGCAAGCGGC GC 3' (Seq. 1 N: 3d)

Oligo 24: 5' GCCGCTTGCA CTCAGCAGCG CAGTCA 3' (Seq. 1 N: 3d)

- heliomicine A6A7A8A9: replacement of the

20 amino acids Asp6-Lys7-Leu8-Ile9 of the sequence ID NO: 1 by 4 alanine amino acids (Ala). By analogy with the sequence encoding the heliomicine of the sequence ID NO: 1, the part GAC-AAG-TTG-ATT encoding the peptide residue Asp-Lys-Leu-Ile is replaced by the sequence 25 GCT-GCT-GCT-GCT encoding the peptide residue Ala-Ala-Ala-Ala. The oligonucleotides 25 and 26 are used as a replacement for the oligonucleotide 1 of Example II and

the oligonucleotides 27 and 28 as a replacement for the oligonucleotide 2.

Oligo 25: 5' AGCTTGGATA AAAGAGCTGC TGCTGCTGGT
AGCTGTGTTT 3' (Seq ID No. 33)

5 Oligo 26: 5' GGGGCGCCG TCAACTACA 3' (Seq ID No. 34)

Oligo 27: 5' CTAGTGTAGT TGACGGCGCC CC 3' (Seq ID No. 35)

Oligo 28: 5' AAACACAGCT ACCAGCAGCA GCAGCTCTTT TATCCA 3'
(Seq ID No. 36)
- heliomicine A24A25L28L29: Two

oligonucleotides (sense and antisense) were necessary
10 to compensate for the absence of a restriction site
between the sequence encoding the peptide residue
consisting of the two amino acids Asn24-Gly25 and the
sequence encoding the peptide residue consisting of the
two amino acids Lys28-Arg29 of the heliomicine of the
15 sequence ID NO: 1. The two oligonucleotide sequences 29
and 30 replace respectively the two oligonucleotide
sequences 3 and 4 of Example II.

Oligo 29: 5' CTAGTGACTG CGCTGCTGAG TGCTTGTGCGC 3'
(Seq ID No. 37)

Oligo 30: 5' GCAACAAGCA CTCAGCAGCG CAGTCA 3'
(Seq ID No. 38)

20 **Production of mutated heliomicine on the
semipreparative scale**

The various mutants of heliomicine are
prepared and purified in the following manner. One of
the transformed yeast clones expressing the mutated
25 heliomicine was cultured at 29°C for 48 h in 50 ml of
selective medium. This preculture was then used to
inoculate 2 l of selective medium and the culture was

carried out for 48 h at 29°C. The yeasts were removed by centrifugation (4000 g, 30 min, 4°C). The supernatant was acidified to pH 3.5 with acetic acid, subjected to a second centrifugation (4000 g, 30 min, 5 4°C) before a first solid phase extraction step.

- first solid phase extraction step on a reversed phase gel: the acidified supernatant is deposited on a C18 reversed phase Sep-Pak Vac 35cc cartridge (Waters Associates, 10 g of phase)

10 equilibrated with acidified water (0.05% TFA). The hydrophilic molecules were removed by washing with acidified water followed by washing with a 15% acetonitrile solution prepared in 0.05% TFA. The elution of the peptide was carried out with a 60% 15 acetonitrile solution prepared in 0.05% TFA. The fraction eluted with 60% acetonitrile was freeze-dried and then reconstituted in sterile ultrapure water before being subjected to the first purification step.

- second solid phase extraction step on a 20 cation-exchange gel: the 60% prepurified fraction containing the mutated heliomicine was reconstituted in 25 mM ammonium acetate solution, pH 3.4. This sample was deposited on a CM cation-exchange Sep-Pak Vac 35cc cartridge (Waters Associates, 10 g of phase)

25 equilibrated with 25 mM ammonium acetate, pH 3.4. The mutated heliomicine is eluted using a 1 M sodium chloride (NaCl) solution prepared in 25 mM ammonium

acetate, pH 3.4. The 1 M NaCl fraction containing the mutated heliomicine is recovered, dried under vacuum, reconstituted with 20 ml of acidified ultrapure water (1% TFA). The mutated heliomicine is then purified by 5 reversed-phase HPLC.

- last purification step by HPLC: the mutated heliomicine was purified to homogeneity by chromatography on a preparative reversed-phase column Aquapo re RP-300 C8 (Brownlee™, 220 x 10 mm, 300 Å), 10 using a biphasic linear gradient of acetonitrile from 2% to 23% over 10 min and from 23% to 33% over 80 min in 0.05% TFA at constant flow rate of 2.5 ml/min. The fraction collected is dried under vacuum, reconstituted with ultrapure water and analysed by MALDI mass 15 spectrometry in order to verify the purity and the identity. The different mutated heliomicines were analysed for their antifungal activity under the conditions described for the reference heliomicine against the following strains: *Neurospora crassa*, 20 *Fusarium culmorum* and *Nectria haematococca*. The activity of the mutants of heliomicine was also evaluated against bacteria. The experimental conditions used are described below.

Test of activity in vitro: measurement of the 25 antibacterial and antifungal activity by microspectrophotometry

This methodology was used for the

determination of the activity spectrum of the peptide and of the minimum inhibitory concentration (MIC) at which the mutated peptide is active. The MIC was expressed as the concentration range [a] - [b] where 5 [a] was the minimum concentration where an onset of growth is observed and [b] the concentration for which no growth was observed. Examples of specific activity of the mutated heliomicine, with respect to bacteria and filamentous fungi, are given in Table 3.

10 The antibacterial activity was detected by a test of inhibition of growth in liquid medium. The bacteria to be tested were suspended in a nutrient medium of the "Poor Broth" type. Preferably, a 1% bactotryptone solution supplemented with 1% NaCl by 15 weight/volume, prepared in demineralized water, is used. 10 μ l of each fraction to be analysed are deposited in microtiter plates in the presence of 90 μ l of culture medium containing the bacteria (at a final concentration equivalent to 1 mOD at 600 nm). The 20 incubation was carried out at 25°C for 12 to 24 hours. The bacterial growth was measured by monitoring absorbance at 600 nm with the aid of a microtiter plate reader spectrophotometer.

- bacteria tested: *Bacillus megaterium*
25 (collection de Institut Pasteur), *Micrococcus luteus* (collection de l'Institut Pasteur), *Staphylococcus aureus* (H. Monteil, Institute of bacteriology,

Strasbourg), *Aerococcus viridans* (H. Monteil, Institute of bacteriology, Strasbourg), and *Escherichia coli* D22 (P.L. Boquet, Centre for nuclear studies, Saclay).

Table 3: Activity of some mutated heliomicines against
5 filamentous fungi and bacteria

Microorganisms	MIC for the mutants of heliomicine (μ m)				
	L28L29	R48	L28L29R48	A6A7A8A9	Helio
Fungi					
<i>Neurospora</i>	0.8-1.6	0.4-0.8	0.8-1.6	1.6-3.1	0.1-0.2
<i>crassa</i>					
<i>Fusarium</i>	3.1-6.2	0.4-0.8	0.8-1.6	3.1-6.2	0.2-0.4
<i>culmorum</i>					
<i>Nectria</i>	3.1-6.2	0.4-0.8	0.8-1.6	ND	0.4-0.8
<i>haematococca</i>					
Bacteria					
<i>Bacillus</i>	50-100	ND	ND	6.2-12.5	ND
<i>megaterium</i>					
<i>Micrococcus</i>	12.5-25	25-50	ND	ND	ND
<i>luteus</i>					
<i>Staphylococcus</i>	ND	ND	ND	ND	ND
<i>aureus</i>					
<i>Aerococcus</i>	ND	ND	ND	12.5-25	ND
<i>viridans</i>					
<i>Escherichia</i>	ND	ND	ND	ND	ND
<i>coli</i> D22					

ND: no activity detected

Example VIII: Study of acute toxicity

Groups of 4 female mice were treated by intravenous injection of solutions of heliomicine (SEQ 5 ID NO 2) in saline solution at doses of 1 and 10 mg/kg. Corresponding solutions of aprotinin as negative control (no effect for the two doses) and mellitin as positive control (100% mortality at 5 days at 10 mg, significant effects at 5 days at 1 mg). No toxicity was 10 demonstrated for the heliomicine solutions at the two doses injected.

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CLAIMS

1. Peptide comprising essentially the peptide sequence of formula (I),

in which:

Xaa is -NH₂ or a peptide residue comprising
10 from 1 to 10 amino acids, preferably from 1 to 6 amino
acids.

Xab is a peptide residue comprising from 1 to 10 amino acids, preferably 10.

Xac is a peptide residue of 3 amino acids

15 Xad is a peptide residue comprising from 1 to
9 amino acids, preferably 9

Xae is a peptide residue comprising from 1 to 7 amino acids, preferably 7

Xaf is a peptide residue of 1 amino acid and

20 Xag is -OH or a peptide residue comprising
from 1 to 5 amino acids, preferably 1 or 2 amino acid

2. Peptide according to claim 1,
characterized in that

Xaa comprises at least one basic amino acid, and/or
25 Xad comprises at least one basic amino acid.

3. Peptide according to claim 2, characterized in that Xad comprises 1, 2, 3 or 4 basic amino acids.

4. Peptide according to either of claims 2
5 and 3, characterized in that the basic amino acids are chosen from lysine, arginine or homoarginine.

5. Peptide according to one of claims 1 to 4, characterized in that Xad represents the following peptide sequence -Lys-Xad'-Xad"-Gly-His-, in which Xad'
10 represents a peptide residue of 1 basic amino acid and Xad" represents a peptide residue comprising from 0 to 5 amino acids, preferably 5.

6. Peptide according to one of claims 1 to 5, characterized in that Xad represents the following
15 peptide sequence -Lys-Arg-Arg-Gly-Tyr-Lys-Gly-Gly-His-.

7. Peptide according to one of claims 1 to 6, characterized in that Xac comprises at least one acidic amino acid, preferably 1.

8. Peptide according to one of claims 1 to
20 7, characterized in that Xac represents the following peptide sequence -Asn-Xac'-Xac"-, in which Xac'
represents a peptide residue of 1 amino acid, and Xac"
represents a peptide residue of 1 acidic amino acid.

9. Peptide according to either of claims 7
25 and 8, characterized in that the acidic amino acids are chosen from glutamic acid (Glu) or aspartic acid (Asp).

10. Peptide according to one of claims 1 to 10, characterized in that Xac represents the following peptide sequence -Asn-Gly-Glu-.

11. Peptide according to one of claims 1 to 5 10, characterized in that

Xaa represents the following peptide sequence Xaa'-Gly-Xaa"- in which Xaa' represents NH₂ or a peptide residue comprising 1 to 9 amino acids, preferably 1 to 5 amino acids, and Xaa" represents a peptide residue comprising

10 at least one amino acid, preferably chosen from Leu, Ile, Val, Pro, Ser or Thr, and/or

Xab represents the following peptide sequence -Val-Xab'-Asp-, in which Xab' represents a peptide residue comprising from 0 to 8 amino acids, preferably 8,

15 and/or

Xae represents the following peptide sequence -Gly-Xae'-Asn-, in which Xae' represents a peptide residue comprising from 0 to 5 amino acids, preferably 5, and/or

20 Xaf represents one of the following amino acids Trp, Phe, Leu, Ile or Val and/or

Xag represents the following peptide sequence -Glu-Xag' in which Xag' represents OH or a variable residue having a sequence comprising from 1 to 4 amino acids,

25 preferably 1 amino acid.

12. Peptide according to one of claims 1 to 11, characterized in that

Xaa represents the following peptide sequence NH₂-Asp-Lys-Leu-Ile-Gly-Ser-, and/or

Xab represents the following peptide sequence -Val-Trp-Gly-Ala-Val-Asn-Tyr-Thr-Ser-Asp-, and/or

5 Xae represents the following peptide sequence -Gly-Ser-Phe-Ala-Asn-Val-Asn-, and/or

Xaf represents the following amino acid -Trp- and/or

Xag represents the following peptide sequence -Glu-Thr-OH.

10 13. Peptide according to one of claims 1 to 12, characterized in that it is represented by the identifier No. 2 (SEQ ID NO 2).

14. Peptide according to one of claims 1 to 13, characterized in that it comprises at either of its 15 ends, or at both ends, peptide residues necessary for its expression and targeting in a host organism.

15. Peptide according to one of claims 1 to 14, characterized in that the cysteine residues of the peptide of formula (I) form at least one intramolecular 20 disulphide bridge.

16. Peptide according to claim 15, characterized in that it comprises 3 disulphide bridges established between the cysteine residues 1 and 4, 2 and 5, and 3 and 6.

25 17. "Peptide-heliomicine" fusion peptide, characterized in that the heliomicine is a peptide defined according to one of claims 1 to 16.

18. Fusion peptide according to claim 17, characterized in that the peptide fused with heliomicine is a signal peptide or a transit peptide.

19. Fusion peptide according to claim 18, 5 characterized in that the transit peptide is the signal peptide of the tobacco PR-1 α gene or the precursor of factor Mat alpha 1 or the signal peptide of the maize polygalacturonase PG1 gene.

20. Fusion peptide according to claim 19, 10 characterized in that it is represented by the sequence identifier No. 1 (SEQ ID NO 1), by the sequence identifier No. 3 (SEQ ID NO 3), or by the sequence identifier No. 18 (SEQ ID NO 18).

21. As a medicament, the peptide according 15 to one of claims 1 to 20.

22. Composition, characterized in that it comprises the peptide according to one of claims 1 to 20 and an appropriate vehicle.

23. Nucleic acid fragment, characterized in 20 that it comprises a nucleic acid sequence encoding a peptide according to one of claims 1 to 20.

24. Nucleic acid fragment according to claim 23, characterized in that it is a nucleotide sequence of the DNA type.

25. Nucleic acid fragment according to claim 24, characterized in that the nucleotide sequence of the DNA type comprises the DNA sequence described by bases 16 to 147 of the sequence identifier No. 1 (SEQ ID NO 1), by the sequence identifier No. 2 (SEQ ID NO 2), by bases 3 to 224 of the sequence identifier No. 3 (SEQ ID NO 3), or by bases 7 to 205 of the sequence identifier No. 18 (SEQ ID NO 18), a homologous sequence or a sequence complementary to the said sequence.

10 26. Chimeric gene comprising a coding sequence as well as heterologous regulatory elements at the 5' and 3' positions capable of functioning in a host organism, in particular plants, characterized in that the coding sequence comprises at least one DNA 15 fragment as defined in claims 23 to 25.

27. Chimeric gene according to claim 26, characterized in that the host organism is a microorganism.

28. Chimeric gene according to claim 26, 20 characterized in that the host organism is chosen from plant cells and plants.

29. Cloning or expression vector for the transformation of a host organism, characterized in that it comprises at least one replication origin and 25 at least one chimeric gene as defined in claims 26 to 28.

30. Transformed host organisms, characterized in that they contain a nucleic acid fragment according to claims 23 to 25, or a chimeric gene according to claims 26 to 28.

5 31. Transformed host organism according to claim 30, characterized in that it includes microorganisms, plant cells or plants.

32. Transformed host organism according to claim 30, characterized in that it is a plant 10 containing transformed cells.

33. Host organism according to claim 32, characterized in that the plant is regenerated from transformed cells.

34. Transformed host organism according to 15 claim 30, characterized in that the microorganism is chosen from bacteria, in particular *E. coli*, yeasts, in particular of the genera *Saccharomyces* or *Kluyveromyces*, *Pichia*, fungi, in particular *Aspergillus*, or baculoviruses.

20 35. Transformed plant cell, characterized in that it contains a nucleic acid fragment according to claims 23 to 25 or a chimeric gene according to claims 26 to 28.

36. Transformed plant, characterized in that 25 it comprises at least one transformed plant cell according to claim 35.

37. Transformed plant according to claim 36, characterized in that it is resistant to diseases caused by *Cercospora*, in particular *Cercospora beticola*, *Cladosporium*, in particular *Cladosporium herbarum*, *Fusarium*, in particular *Fusarium culmorum* or *Fusarium graminearum*, or by *Phytophthora*, in particular *Phytophthora cinnamomi*.

38. Transformed plant, characterized in that it is derived from the cultivation and/or crossing of 10 the plants according to either of claims 36 and 37.

39. Seeds of transformed plants according to one of claims 36 to 38.

40. Method of transforming host organisms, in particular plant cells or plants, characterized in 15 that at least one nucleic acid fragment according to claims 23 to 25 or a chimeric gene according to one of claims 26 to 28 is inserted into the said host organism.

41. Method according to claim 40, 20 characterized in that the host organism is a plant cell or a plant.

42. Method according to claim 41, characterized in that a plant is regenerated from the plant cell or from the transformed plant.

43. Method of cultivating transformed plants according to one of claims 36 to 38, characterized in that it consists in planting the seeds of the said transformed plants in a plot of a field appropriate for 5 cultivating the said plants, in applying to the said plot of the said field an agrochemical composition, without substantially affecting the said seeds or the said transformed plants, then in harvesting the cultivated plants when they arrive at the desired 10 maturity and optionally in separating the seeds from the harvested plants.

44. Method of cultivation according to claim 33, characterized in that the agrochemical composition comprises at least one active product having at least 15 one fungicidal and/or bactericidal activity.

45. Method of cultivation according to claim 44, characterized in that the active product exhibits an activity which is complementary to that of the peptide according to one of claims 1 to 20.

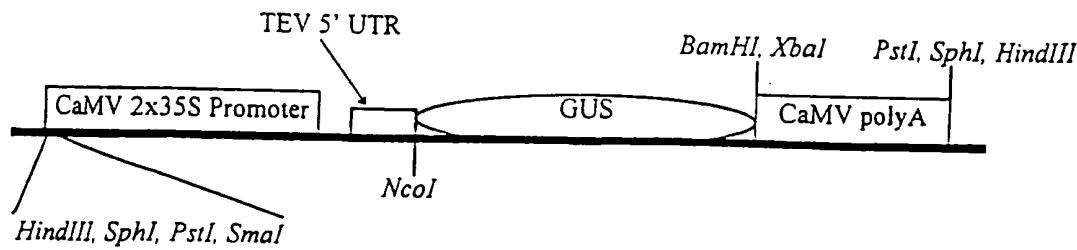
20 46. Method of preparing heliomicine defined according to one of claims 1 to 20, characterized in that it comprises the steps of culturing a transformed organism according to one of claims 30 to 34 in an appropriate culture medium, followed by the extraction 25 and total or partial purification of the heliomicine obtained.

1/2



Fig. 1

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Fig. 2

2/2

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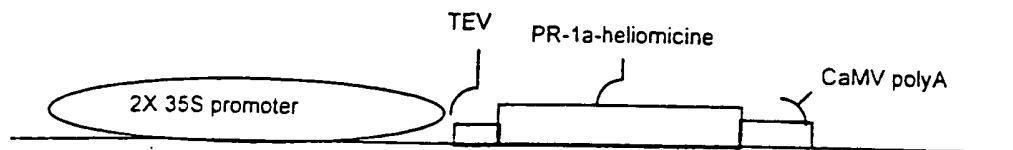


Fig. 3

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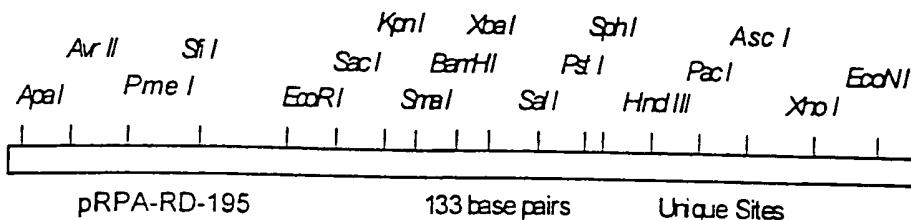
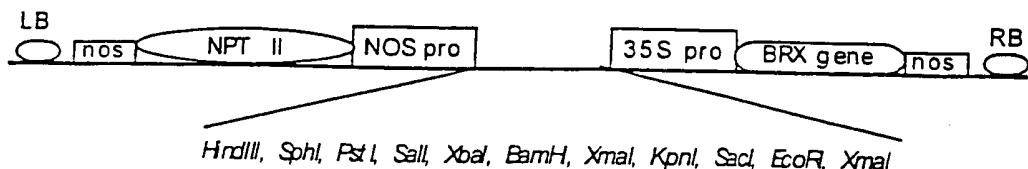


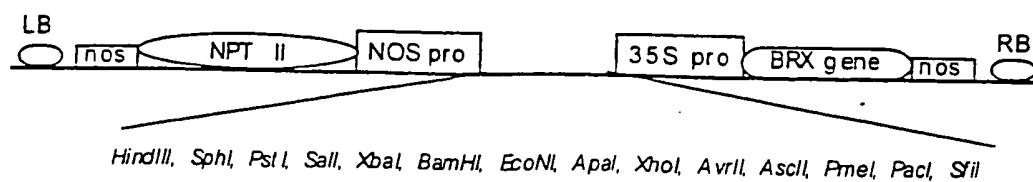
Fig. 4

15



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Fig. 5



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Fig. 6

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: RHONE-POULENC AGROCHIMIE
- (B) STREET: 14-20 Rue Pierre BAIZET
- (C) CITY: LYON
- (E) COUNTRY: France
- (F) POSTAL CODE: 69009

(ii) TITLE OF INVENTION: Gene encoding heliomicine, protein obtained, vector containing it, transformed organisms obtained and method of preparation

(iii) NUMBER OF SEQUENCES: 20

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 147 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..147

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AGC TTG GAT AAA AGA GAC AAG TTG ATT GGC AGC TGT GTT TGG GGC GCC	48
Ser Leu Asp Lys Arg Asp Lys Leu Ile Gly Ser Cys Val Trp Gly Ala	
1 5 10 15	
GTC AAC TAC ACT AGT GAC TGC AAC GGC GAG TGC AAG CGC CGC GGT TAC	96
Val Asn Tyr Thr Ser Asp Cys Asn Gly Glu Cys Lys Arg Arg Gly Tyr	
20 25 30	
AAG GGT GGC CAT TGT GGA TCC TTC GCT AAC GTT AAC TGT TGG TGT GAA	144
Lys Gly His Cys Gly Ser Phe Ala Asn Val Asn Cys Trp Cys Glu	
35 40 45	
ACC	147
Thr	
49	

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 169 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..132

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GAT AAG CTT ATC GGT TCC TGC GTG TGG GGT GCT GTG AAC TAC ACT TCC	48
Asp Lys Leu Ile Gly Ser Cys Val Trp Gly Ala Val Asn Tyr Thr Ser	
1 5 10 15	
GAT TGC AAC CGT GAG TGC AAG AGG AGG GGT TAC AAG GGT GGT CAC TGC	96
Asp Cys Asn Gly Glu Cys Lys Arg Arg Gly Tyr Lys Gly Gly His Cys	
20 25 30	
GGT TCC TTC GCT AAC GTG AAC TGC TGG TGC GAG ACT TGAGAGCTCG	142
Gly Ser Phe Ala Asn Val Asn Cys Trp Cys Glu Thr	
35 40	
GGGAGGGCGAA CGTGTGACG GATCCGG	169

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 261 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

(A) NAME/KEY: CDS

0

(B) LOCATION: 3..224

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CC ATG GGT TTC GTG CTT TTC TCT CAG CTT CCA TCT TTC CTT CTT GTG	47
Met Gly Phe Val Leu Phe Ser Gln Leu Pro Ser Phe Leu Leu Val	
1 5 10 15	
TCT ACT CTT CTT CTT TTC CTT GTG ATC TCT CAC TCT TGC CGT GCC GAT	95
Ser Thr Leu Leu Leu Phe Leu Val Ile Ser His Ser Cys Arg Ala Asp	
20 25 30	
AAG CTT ATC GGT TCC TGC GTG TGG GGT GCT GTG AAC TAC ACT TCC GAT	143
Lys Leu Ile Gly Ser Cys Val Trp Gly Ala Val Asn Tyr Thr Ser Asp	
35 40 45	
TGC AAC GGT GAG TGC AAG AGG AGG GGT TAC AAG GGT GGT CAC TGC GGT	191
Cys Asn Gly Glu Cys Lys Arg Arg Gly Tyr Lys Gly Gly His Cys Gly	
50 55 60	
TCC TTC GCT AAC GTG AAC TGC TGG TGC GAG ACT TGAGAGCTCG GCGAGGCGAA	244
Ser Phe Ala Asn Val Asn Cys Trp Cys Glu Thr	
65 70	
CGTGTCGACG GATCCGG	261

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 120 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 12..101

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCGTCGACGC G ATG GGT TTC GTG CTT TTC TCT CAG CTT CCA TCT TTC CTT	50
Met Gly Phe Val Leu Phe Ser Gln Leu Pro Ser Phe Leu	
1 5 10	
CTT GTG TCT ACT CTT CTT TTC CTT GTG ATC TCT CAC TCT TGC CGT	98
Leu Val Ser Thr Leu Leu Phe Leu Val Ile Ser His Ser Cys Arg	
15 20 25	
GCT GGAGACGCGA ATTACACACA	129
Ala	
30	

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 75 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

- (A) DESCRIPTION: /desc = "synthetic oligonucleotide 7"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:	
GCGTCGACGC GATGGGTTTC GTGCTTTCT CTCAGCTTCC ATCTTCCTT CTTGTGTCTA	60
CTCTTCTTCT TTTCC	75

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 72 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: /desc = "synthetic
oligonucleotide 8"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:
TCGCCGGCAC GGCAAGAGTA AGAGATCACA AGGAAAAGAA GAAGAGTAGA CACAAGAAGG 60
AAAGATGGAA GC 72

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 80 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: /desc = "synthetic
oligonucleotide 9"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:
GATAAGCTTA TCGGTTCTG CGTGTGGGT GCTGTGAAC TACACTTCCGA TTGCAACGGT 60
GAGTGCAAGA GGAGGGGTTA 80

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 109 base pairs
- (B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: /desc = "synthetic
oligonucleotide 10"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:
CCGGATCCGT CGACACGTTG GCCTCGCCGA GCTCTCAAGT CTCGCACCAAG CAGTTCACGT 60
TAGCGAAGGA ACCGCAGTGA CCACCCCTTGT AACCCCTCCT CTTGCACTC 109

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 85 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: /desc = "synthetic
oligonucleotide 11"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:
AGGGCCCCCT AGGGTTTAAA CGGCCAGTCA GGCGAATTG GAGCTCGGTA CCCGGGGATC 60
CTCTAGAGTC GACCTGCAGG CATGC 85

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 66 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: /desc = "synthetic
oligonucleotide 12"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:
CCCTGAACCA GGCTCGAGGG CGCGCCTTAA TTAAAAGCTT GCATGCCTGC AGGTCGACTC 60
TAGAGG 66

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 93 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: /desc = "synthetic
oligonucleotide 13"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:
CCGGCCAGTC AGGCCACACT TAATTAAGTT TAAACGCGGC CCCGGCGCGC CTAGGTGTGT 60
GCTCGAGGGC CCAACCTCAG TACCTGGTTC AGG 93

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 93 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: /desc = "synthetic
oligonucleotide 14"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CCGGCCTGAA CCAGGTACTG AGGTTGGGCC CTCGAGCACA CACCTAGGCG CGCCGGGGCC 60
GCGTTAAC TTAATTAAGT GTGGCCTGAC TGG 93

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: /desc = "synthetic
oligonucleotide 15"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GGTCTAGAAC GGCCTGCACC AACAAACGCCA TGAGGGCCCT CTTCCCTCTC 50

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: /desc = "synthetic
oligonucleotide 16"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CCGAATTCGG CGCCGTGCAC GATGCAGAAG AGCACGAGGA GGAAGAGGGC

50

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 81 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

- (A) NAME/KEY: CDS

(B) LOCATION: 7..73

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

TCTAGA ATG GCC TGC ACC AAC AAC GCC ATG AGG GCC CTC TTC CTC CTC
Met Ala Cys Thr Asn Asn Ala Met Arg Ala Leu Phe Cys Ile
1 5 10

48

CTG CTC TTC TGC ATC GTG CAC GGC GCCGAATTC
Val Leu Phe Cys Ile Val His Gly
15 20

81

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: /desc = "synthetic
oligonucleotide 17"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GATAAGCTTA TCGGTTCCCTG CGTG

24

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: /desc = "synthetic
oligonucleotide 18"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GGCTCGAGTC AAGTCTCGCA CCAGCAGTTC AC

32

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 213 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 7..205

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

TCTAGA ATG GCC TGC ACC AAC AAC GCC ATG AGG GCC CTC TTC CTC CTC

48

Met Ala Cys Thr Asn Asn Ala Met Arg Ala Leu Phe Cys Ile			
1	5	10	
CTG CTC TTC TGC ATC GTG CAC GGC GAT AAG CTT ATC GGT TCC TGC GTG		96	
Val Leu Phe Cys Ile Val His Gly Asp Lys Leu Ile Gly Ser Cys Val			
15	20	25	30
TGG GGT GCT GTG AAC TAC ACT TCC GAT TGC AAC GGT GAG TGC AAG AGG		144	
Trp Gly Ala Val Asn Tyr Thr Ser Asp Cys Asn Gly Glu Cys Lys Arg			
35	40	45	
AGG GGT TAC AAG GGT GGT CAC TGC GGT TCC TTC GCT AAC GTG AAC TGC		192	
Arg Gly Tyr Lys Gly Gly His Cys Gly Ser Phe Ala Asn Val Asn Cys			
50	55	60	
TGG TGC GAG ACT TGACTCGAG		213	
Trp Cys Glu Thr			
65			

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 838 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

- (A) NAME/KEY: CsVMV promoter
- (B) LOCATION: 7..532

(ix) FEATURE

- (A) NAME/KEY: multiple cloning site
- (B) LOCATION: 533..568

(ix) FEATURE

(A) NAME/KEY: terminator

(B) LOCATION: 569..832

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

AAGCTTCCAG AAGGTAATTA TCCAAGATGT AGCATCAAGA ATCCAATGTT TACGGGAAAA	60
ACTATGGAAG TATTATGTGA GCTCAGCAAG AAGCAGATCA ATATGCGGCA CATATGCAAC	120
CTATGTTCAA AAATGAAGAA TGTACAGATA CAAGATCCTA TACTGCCAGA ATACGAAGAA	180
GAATACGTAG AAATTGAAAA AGAAGAACCA GGCGAAGAAA AGAACATTTGA AGACGTAAGC	240
ACTGACGACA ACAATGAAAAA GAAGAAGATA AGGTGGTGA TTGTGAAAGA GACATAGAGG	300
ACACATGTAATGGTGGAAAT GTAGGGCGG AAAGTAACCT TATCACAAAG GAATCTTATC	360
CCCCACTACT TATCCTTTA TATTTTCCG TGTCATTTT GCCCTTGAGT TTTCCTATAT	420
AAGGAACCAA GTTCCGCATT TGTGAAAACA AGAAAAAAATT TGGTGTAAGC TATTTTCTTT	480
GAAGTACTGA GGATACAAC TCAAGAGAAA TTGTAAGTTT GTAGATCTCG ATTCTAGAAG	540
GCCTGAATTC GAGCTCGGTA CCGGATCCAA TTCCCGATCG TTCAACATT TGGCAATAAA	600
TTTCTTAAG ATTGAATCCT GTTCCGGTC TTGCGATGAT TATCATATAA TTCTGTTGA	660
ATTACGTTAA GCATGTAATA ATTAACATGT AATGCATGAC GTTATTTATG AGATGGGTTT	720
TTATGATTAG AGTCCCGCAA TTATACATTT AATACGCGAT AGAAAACAAA ATATAGCGCG	780
CAAACCTAGGA TAAATTATCG CGCGCGGTGT CATCTATGTT ACTAGATCGG GGATCGAT	838

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1036 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

(A) NAME/KEY: CsVMV promoter

(B) LOCATION: 7..532

(ix) FEATURE

(A) NAME/KEY: CDS

(B) LOCATION: 539..736

(ix) FEATURE

(A) NAME/KEY: nos terminator

(B) LOCATION: 767..1030

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

AAGCTTCCAG AAGGTAATTA TCCAAGATGT AGCATCAAGA ATCCAATGTT TACGGGAAAA	60
ACTATGGAAG TATTATGTGA GCTCAGCAAG AACCAGATCA ATATGCCGA CATATGCAAC	120
CTATGTTCAA AAATGAAGAA TGTACAGATA CAAGATCCTA TACTGCCAGA ATACGAAGAA	180
GAATACTAG AAATTGAAAA AGAAGAACCA GGCAGAGAAA AGAATCTTGA AGACGTAAGC	240
ACTGACGACA ACAATGAAAA GAAGAAGATA AGGTCCGTGA TTGTGAAAGA GACATAGAGG	300
ACACATGTAA GGTGGAAAAT GTAAGGGCGG AAAGTAACCT TATCACAAAG GAATCTTATC	360
CCCCCACTACT TATCCTTTTA TATTTTCCG TGTCATTTT GCCCTTGAGT TTTCTATAT	420
AAGGAACCAA GTTCGGCATT TGTGAAAACA AGAAAAAATT TGGTGTAAGC TATTTCTTT	480
GAAGTACTGA GGATACAAC TCAAGAGAAA TTGTAAGTT GTAGATCTCG ATTCTAGA	538

ATG GCC TGC ACC AAC AAC GCC ATG AGG GCC CTC TTC CTC CTC GTG CTC	586
Met Ala Cys Thr Asn Asn Ala Met Arg Ala Leu Phe Leu Leu Val Leu	
1 5 10 15	
TTC TGC ATC GTG CAC GGC GAT AAG CTT ATC GGT TCC TGC GTG TGG GGT	634
Phe Cys Ile Val His Gly Asp Lys Leu Ile Gly Ser Cys Val Trp Gly	
20 25 30	
GCT GTG AAC TAC ACT TCC GAT TGC AAC GGT GAG TGC AAG AGG AGG GGT	682
Ala Val Asn Tyr Thr Ser Asp Cys Asn Gly Glu Cys Lys Arg Arg Gly	
35 40 45	
TAC AAG GGT GGT CAC TGC GGT TCC TTC GCT AAC GTG AAC TGC TGG TGC	730
Tyr Lys Gly Gly His Cys Gly Ser Phe Ala Asn Val Asn Cys Trp Cys	
50 55 60	
GAG ACT TGACTCGAGG GGGGGCCCGG TACCGGATCC AATTCCCGAT CGTTCAAACA	786
Glu Thr	
65	
TTTGGCAATA AAGTTTCTTA AGATTGAATC CTGTTGCCGG TCTTGCATG ATTATCATAT	846
AATTCTGTT GAATTACGTT AAGCATGTA TAATTAACAT GTAATGCATG ACGTTATTAA	906
TGAGATGGGT TTTTATGATT AGAGTCCCGC AATTATAACAT TTAATACGCG ATAGAAAACA	966
AAATATAGCG CGCAAACCTAG GATAAATTAT CGCGCGCGGT GTCATCTATG TTACTAGATC	1026
GGGGATCGAT	1036

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 52 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic
oligonucleotide 1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

AGCTTGGATA AAAGAGACAA GTTGATTGGC AGCTGTGTTT GGGGCGCCGT CA 52

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 56 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic
oligonucleotide 2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

AGTGTAGTTG ACGGCGCCCC AAACACAGCT GCCAATCAAC TTGTCTCTTT TATCCA 56

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 52 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic
oligonucleotide 3"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

ACTACACTAG TGACTGCAAC GGCGAGTGCA AGCGCCGCGG TTACAAGGGT GG 52

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 52 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic
oligonucleotide 4"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CACAATGGCC ACCCTTGTAA CCGCGGCCGCT TGCACTCGCC GTTGCAGTCA CT 52

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 56 base pairs

- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic
oligonucleotide 5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CCATTGTGGA TCCTTCGCTA ACGTTAACTG TTGGTGTGAA ACCTGATAGG TCGACA 56

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 52 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic
oligonucleotide 6"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GATCTGTCGA CCTATCAGGT TTCACACCAA CAGTTAACGT TAGCGAAGGA TC 52

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

- (A) DESCRIPTION:/desc = "synthetic oligonucleotide 19"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GATCCTTCGC TAACGTTAAC TGTTGGTGTA GAACCTGATA GG

42

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

- (A) DESCRIPTION:/desc = "synthetic

oligonucleotide 20"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

TCGACCTATC AGGTTCTACA CCAACAGTTA ACGTTAGCGA AG 42

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic
oligonucleotide 21"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

CTAGTGACTG CAACGGCGAG TGCTTGTGCGC 32

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic
oligonucleotide 22"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

GCAACAAGCA CTCGCCGTTG CAGTCA

26

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic
oligonucleotide 23"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

CTAGTGACTG CGCTGCTGAG TGCAAGCGGC GC

32

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic
oligonucleotide 24"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

GCGGCTTGCA CTCAGCAGCG CAGTCA

26

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic
oligonucleotide 25"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

AGCTTGGATA AAAGAGCTGC TGCTGCTGGT AGCTGTGTTT 40

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic
oligonucleotide 26"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

GGGGCGCCGT CAACTACA 18

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic
oligonucleotide 27"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

CTAGTGTAGT TGACGGCGCC CC

22

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:/desc = "synthetic
oligonucleotide 28"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

AAACACAGCT ACCAGCAGCA GCAGCTTT TATCCA

36

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

- (A) DESCRIPTION:/desc = "synthetic oligonucleotide 29"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

CTAGTGACTG CGCTGCTGAG TGCTTGTGCGC

32

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

- (A) DESCRIPTION:/desc = "synthetic oligonucleotide 30"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

GCAACAAGCA CTCAGCAGCG CAGTCA

INTERNATIONAL SEARCH REPORT

International Application No
PCT/FR 99/00843

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/435 C12N15/82 A61K38/17 C12P21/02
C12N15/62 C12N15/81

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FR 2 695 392 A (CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE) 11 March 1994 (1994-03-11) page 1, line 33 - page 7, line 2	1-4, 8, 11, 21, 22, 46
Y	---	13, 14, 25
X	WO 90 11770 A (CALGENE INC) 18 October 1990 (1990-10-18)	1-4, 8, 11, 17, 18, 21-24, 28-46
	page 1 - page 19	---
X	FR 2 725 992 A (RHONE POULENC AGROCHIMIE) 26 April 1996 (1996-04-26) cited in the application the whole document	1-4, 8, 11, 21, 22
	---	-/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

27 August 1999

13/09/1999

Name and mailing address of the ISA

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Authorized officer

De Kok, A

INTERNATIONAL SEARCH REPORT

International Application No
PCT/FR 99/00843

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHUNG K T ET AL: "Antibacterial factors in immune hemolymph from heliothis virescens larvae" ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 96, no. 0, 19 May 1996 (1996-05-19), page 275 XP002089180 WASHINGTON US abstract ---	13,14,25
A	DE 22 12 854 A (WSESOJUSNYJ NAUTSCHNO) 2 November 1972 (1972-11-02) the whole document ---	1,21,22, 46
A	WO 97 30082 A (RHONE POULENC AGROCHIMIE) 21 August 1997 (1997-08-21) the whole document & FR 2 745 004 A cited in the application ---	1,21,22
A	EP 0 307 841 A (THE GENERAL HOSPITAL CORP.) 22 March 1989 (1989-03-22) the whole document ---	17,19, 20,23, 25,26, 29,30, 35,36
A	EP 0 607 080 A (TRANSGENE SA) 20 July 1994 (1994-07-20) page 2, line 28 - line 53 ---	17-19
A	HOFFMANN J A ET AL.: "Insect defensins: inducible antibacterial peptides" IMMUNOLOGY TODAY., vol. 13, no. 10, 1992, pages 411-415, XP002089181 CAMBRIDGE GB the whole document ---	1-46
P,X	LAMBERTY M ET AL.: "Insect immunity - Isolation from the lepidopteran Heliothis virescens of a novel insect defensin with potent antifungal activity" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 274, no. 14, 2 April 1999 (1999-04-02), pages 9320-9326, XP002112857 BALTIMORE, US ISSN: 0021-9258 the whole document -----	1-25

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/FR 99/00843

Patent document cited in search report	Publication date	Patent family member(s)			Publication date
FR 2695392	A 11-03-1994	NONE			
WO 9011770	A 18-10-1990	CA 2030779 A	12-10-1990	EP 0425616 A	08-05-1991
FR 2725992	A 26-04-1996	NONE			
DE 2212854	A 02-11-1972	CH 568387 A	31-10-1975	FR 2130267 A	03-11-1972
		GB 1355163 A	05-06-1974		
WO 9730082	A 21-08-1997	FR 2745004 A	22-08-1997	AU 1884397 A	02-09-1997
		CA 2245518 A	21-08-1997	CN 1216047 A	05-05-1999
		EP 0882063 A	09-12-1998	PL 328579 A	01-02-1999
EP 0307841	A 22-03-1989	AU 2487788 A	17-04-1989	WO 8902437 A	23-03-1989
EP 0607080	A 20-07-1994	FR 2700338 A	13-07-1994		

RAPPORT DE RECHERCHE INTERNATIONALE

Dem: Internationale No
PCT/FR 99/00843

A. CLASSEMENT DE L'OBJET DE LA DEMANDE
CIB 6 C12N15/12 C07K14/435 C12N15/82 A61K38/17 C12P21/02
C12N15/62 C12N15/81

Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

B. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE

Documentation minimale consultée (système de classification suivi des symboles de classement)
CIB 6 C07K C12N

Documentation consultée autre que la documentation minimale dans la mesure où ces documents relèvent des domaines sur lesquels a porté la recherche

Base de données électronique consultée au cours de la recherche internationale (nom de la base de données, et si réalisable, termes de recherche utilisés)

C. DOCUMENTS CONSIDÉRÉS COMME PERTINENTS

Catégorie *	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
X	FR 2 695 392 A (CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE) 11 mars 1994 (1994-03-11) page 1, ligne 33 - page 7, ligne 2	1-4, 8, 11, 21, 22, 46
Y	---	13, 14, 25
X	WO 90 11770 A (CALGENE INC) 18 octobre 1990 (1990-10-18)	1-4, 8, 11, 17, 18, 21-24, 28-46
	page 1 - page 19	---
X	FR 2 725 992 A (RHONE POULENC AGROCHIMIE) 26 avril 1996 (1996-04-26) cité dans la demande le document en entier	1-4, 8, 11, 21, 22
	---	-/-

Voir la suite du cadre C pour la fin de la liste des documents

Les documents de familles de brevets sont indiqués en annexe

* Catégories spéciales de documents cités:

- "A" document définissant l'état général de la technique, non considéré comme particulièrement pertinent
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- "L" document pouvant poser un doute sur une revendication de priorité ou cité pour déterminer la date de publication d'une autre citation ou pour une raison spéciale (telle qu'indiquée)
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"X" document particulièrement pertinent: l'invention revendiquée ne peut être considérée comme nouvelle ou comme impliquant une activité inventive par rapport au document considéré isolément

"Y" document particulièrement pertinent: l'invention revendiquée ne peut être considérée comme impliquant une activité inventive lorsque le document est associé à un ou plusieurs autres documents de même nature, cette combinaison étant évidente pour une personne du métier

"S" document qui fait partie de la même famille de brevets

Date à laquelle la recherche internationale a été effectivement achevée

Date d'expédition du présent rapport de recherche internationale

27 août 1999

13/09/1999

Nom et adresse postale de l'administration chargée de la recherche internationale
Office Européen des Brevets, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Fonctionnaire autorisé

De Kok, A

RAPPORT DE RECHERCHE INTERNATIONALE

Demande Internationale No
PCT/FR 99/00843

C.(suite) DOCUMENTS CONSIDERES COMME PERTINENTS		
Categorie	Identification des documents cités, avec le cas échéant, l'indication des passages pertinents	no. des revendications visées
Y	CHUNG K T ET AL: "Antibacterial factors in immune hemolymph from heliothis virescens larvae" ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 96, no. 0, 19 mai 1996 (1996-05-19), page 275 XP002089180 WASHINGTON US abrégué	13,14,25
A	DE 22 12 854 A (WSESOJUSNYJ NAUTSCHNO) 2 novembre 1972 (1972-11-02) le document en entier	1,21,22, 46
A	WO 97 30082 A (RHONE POULENC AGROCHIMIE) 21 août 1997 (1997-08-21) le document en entier & FR 2 745 004 A cité dans la demande	1,21,22
A	EP 0 307 841 A (THE GENERAL HOSPITAL CORP.) 22 mars 1989 (1989-03-22)	17,19, 20,23, 25,26, 29,30, 35,36
	le document en entier	
A	EP 0 607 080 A (TRANSGENE SA) 20 juillet 1994 (1994-07-20) page 2, ligne 28 - ligne 53	17-19
A	HOFFMANN J A ET AL.: "Insect defensins: inducible antibacterial peptides" IMMUNOLOGY TODAY., vol. 13, no. 10, 1992, pages 411-415, XP002089181 CAMBRIDGE GB le document en entier	1-46
P,X	LAMBERTY M ET AL.: "Insect immunity - Isolation from the lepidopteran Heliothis virescens of a novel insect defensin with potent antifungal activity" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 274, no. 14, 2 avril 1999 (1999-04-02), pages 9320-9326, XP002112857 BALTIMORE, US ISSN: 0021-9258 le document en entier	1-25

RAPPORT DE RECHERCHE INTERNATIONALE

Renseignements relatifs aux membres de familles de brevets

Dem: Internationale No
PCT/FR 99/00843

Document brevet cité au rapport de recherche		Date de publication	Membre(s) de la famille de brevet(s)			Date de publication
FR 2695392	A	11-03-1994	AUCUN			
WO 9011770	A	18-10-1990	CA	2030779 A	12-10-1990	
			EP	0425616 A	08-05-1991	
FR 2725992	A	26-04-1996	AUCUN			
DE 2212854	A	02-11-1972	CH	568387 A	31-10-1975	
			FR	2130267 A	03-11-1972	
			GB	1355163 A	05-06-1974	
WO 9730082	A	21-08-1997	FR	2745004 A	22-08-1997	
			AU	1884397 A	02-09-1997	
			CA	2245518 A	21-08-1997	
			CN	1216047 A	05-05-1999	
			EP	0882063 A	09-12-1998	
			PL	328579 A	01-02-1999	
EP 0307841	A	22-03-1989	AU	2487788 A	17-04-1989	
			WO	8902437 A	23-03-1989	
EP 0607080	A	20-07-1994	FR	2700338 A	13-07-1994	